DOI: 10.2478/s11686-010-0045-z

Acta Parasitologica, 2010, 55(4), 327–339; ISSN 1230-2821

VERSITA

Varialvus gen. nov. (Digenea, Cryptogonimidae), from species of Lutjanidae (Perciformes) off the Great Barrier Reef, New Caledonia and the Maldives

Terrence L. Miller[1,2]*, Rodney A. Bray[3], Jean-Lou Justine[4,5] and Thomas H. Cribb[6]

[1]Biodiversity Program, Queensland Museum, PO Box 3300, South Brisbane, Queensland, Australia; [2]School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Queensland 4072, Australia; [3]Department of Zoology, Natural History Museum, Cromwell Road, London SW7 5BD, UK; [4]UMR 7138 Systématique, Adaptation, Évolution, Muséum National d'Histoire Naturelle, 57, rue Cuvier, 75231 Paris cedex 05, France; [5]Aquarium des Lagons, B.P. 8185, 98807 Nouméa, Nouvelle-Calédonie; [6]School of Biological Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia

Abstract

A survey of the cryptogonimid trematode fauna infecting Indo-West Pacific Lutjanidae (Perciformes) revealed the presence of four new species whose morphological and genetic differences relative to all other known cryptogonimids warrants the proposal of a new genus, *Varialvus* gen. nov. Species of this new genus were recovered from sites off Heron and Lizard Islands on the Great Barrier Reef, Australia, New Caledonia and Rasdhoo Atoll, Maldives. *Varialvus* gen. nov. is distinguished from all other cryptogonimid genera by the combination of a fusiform to oval body, the relatively small number of large oral spines, a median ovary which is relatively condensed and highly lobed, opposite to slightly oblique testes, uterine loops that are restricted to the hindbody and extend well posterior to the testes, and vitelline follicles that are mainly in the forebody but may extend from the anterior margin of the ovary to anterior to the intestinal bifurcation. Bayesian inference analysis of partial large subunit (LSU) rDNA sequence data for these species revealed that they formed a monophyletic clade, despite *V. charadrus* sp. nov. having a distinctly muscular gonotyl, which based on morphological characters alone may have warranted placement in a separate genus in the absence of DNA sequence data. At least one species of *Varialvus* gen. nov. is apparently widespread in the Indo-West Pacific. Three species, *V. lacertus* sp. nov., *V. jenae* sp. nov. and *V. angustus* sp. nov. have each been found at only one locality, but *V. charadrus* sp. nov. was recovered from lutjanids off the Great Barrier Reef, New Caledonia and the Maldives, demonstrating a biogeographic range of at least 10,000 kilometres. *Siphoderina lutjani* (Saoud, Ramadan et Al Kawari, 1988) Miller et Cribb, 2008 is transferred here as *V. lutjani* (Saoud, Ramadan et Al Kawari, 1988) n. comb. based on morphological and host group agreement with species of *Varialvus* gen. nov.

Keywords

Trematoda, Digenea, Cryptogonimidae, Lutjanidae, *Varialvus*, Great Barrier Reef, Maldives, New Caledonia

Introduction

The Cryptogonimidae Ward, 1917 is a large family of digenetic trematodes distributed globally in freshwater and marine teleosts, amphibians and reptiles (Miller and Cribb 2008b). Recent surveys have suggested that cryptogonimids are extraordinarily concentrated and rich in fishes of the Lutjanidae and Haemulidae in the Indo-Pacific (Velasquez 1961, 1975; Manter 1963; Durio and Manter 1969; Yamaguti 1970; Miller and Cribb 2005, 2007a, c, d, 2008a, 2009; Miller *et al*. 2009a). A continued study of the cryptogonimid fauna of coral reef fishes from Australia, New Caledonia and the Maldives revealed the presence of a new genus infecting species of *Lutjanus* at these localities. We complement our morphologically based taxonomic approach to the taxa recovered here with analysis of genetic data from the internal transcribed spacers (ITS1 and ITS2), 5.8S and large subunit (LSU) nuclear ribosomal DNA regions to explore the phylogenetic relationships among these taxa, their host specificity and geographic distributions among the localities where these species were recovered. Large subunit rDNA data for the taxa reported here were aligned with data available from previously reported cryptogonimids (Miller and Cribb 2007a, c, d, 2008a, 2009; Miller *et al*. 2009a, b) and analysed using Bayesian inference analy-

*Corresponding author: terrence.miller@uqconnect.edu.au

sis to explore relationships among individuals of this new genus and intergeneric relationships among these species.

Materials and methods

Host and parasite collection

Fishes were collected by baited line or spear from the following localities: Lizard Island (14°40′S; 145°27′E) in the northern Great Barrier Reef, Queensland, Australia; Baie Maa, New Caledonia (22°13′S, 166°20′E); Reef near La Régnière, off Nouméa, New Caledonia (22°19′S, 166°18′E), Off Pointe Bovis, New Caledonia (22°14′S, 166°20′E) and Rasdhoo Atoll (4°16′N; 72°58′E), Maldives. Fish were killed by neural pithing and the intestine was immediately removed, washed in vertebrate saline (0.85%), and examined for the presence of endohelminths. Digeneans were collected live and immediately fixed in hot saline according to the protocol of Cribb and Bray (2010). Specimens for morphological analysis were then stored in 10% formalin or 70% ethanol and specimens for DNA extraction and analysis were stored in 95–100% ethanol at –20°C.

Morphological samples

Preserved digenean specimens for morphological analysis were washed in freshwater and placed in Mayer's haematoxylin or Mayer's paracarmine for staining. The specimens were overstained and then destained by placing them in a 50% acid-alcohol or a 1.0% HCl-H_2O solution, which was subsequently neutralised in a 0.5–1.0% ammonium hydroxide solution. Stained specimens were then dehydrated through a graded series of ethanol for at least half an hour at each dehydration step, cleared in methyl salicylate or beechwood creosote and mounted in Canada balsam. Drawings were made with the aid of a drawing tube. Measurements were made using an ocular micrometer or Digicad Plus digitising tablet and Carl Zeiss KS100 software adapted by Imaging Associates, and are quoted in micrometres with the mean followed by the range in parentheses. For two-dimensional measurements, length is given before breadth.

Principal components analyses (PCA) were performed on morphometric data using the software PAST (Hammer *et al*. 2001). Each measured morphological character for every individual included in the descriptions below was used in the PCA analysis. Scatterplots were drawn to indicate the position of individual specimens on the axes (Principal component axis 1 *vs* 2). Analyses were bootstrap replicated (n = 1,000) and 95% confidence intervals drawn to show potential separation. Discriminant analysis was also conducted on morphometric data in order to determine which morphological character contributed the most to observed variation between groups or species using Systat 11 software (Systat Software Inc. Chicago, Illinois). Voucher specimens were deposited in the Natural History Museum (NHM), London, the Muséum National d'Histoire Naturelle (MNHN), Paris, or the Queensland Museum (QM), Brisbane, Australia. Line drawings of the specimens reported here were deposited in Morphbank (http://www.morphbank.net), with accession numbers provided below. A key to the species of *Varialvus* gen. nov. is also provided after the species descriptions for reference.

Molecular sample processing

Total genomic DNA from species of *Varialvus* gen. nov. was isolated by a standard proteinase K and phenol:chloroform extraction procedure (Sambrook and Russell 2001). Amplification of the LSU rDNA region was performed with the primers LSU5 (5'-TAGGTCGACCCGCTGAAYTTAAGCA-3' Littlewood *et al*. 2000) and ECD2 (5'-CCTTGGTCCGTGTTTCAAGACGGG-3' Littlewood *et al*. 2000), the ITS1 region with the primers BD1 (5'-GTCGTAACAAGGTTTCCGTA-3' Bowles and McManus 1993) and 4S (5'-TCTAGATGCGTTCGAARTGTCGATG-3' Bowles and McManus 1993) and the ITS2 region with the primers 3S (5'-GGTACCGGTGGATCACGTGGCTAGTG-3' Morgan and Blair 1995) and ITS2.2 (5'-CCTGGTTAGTTTCTTTTCCTCCGC-3' Cribb *et al*. 1998). PCR was conducted for all rDNA regions with a total volume of 20 µl consisting of approximately 10 ng of template DNA, 0.75 µl of each primer (10 pmols), 1.6 µl $MgCl_2$, 2 µl of 10 × reaction buffer, 0.8 µl deoxyribonucleotide triphosphate (dNTP) (each 2.5 mM), and 0.25 µl of Taq DNA polymerase. Amplifications of the LSU, ITS1 and ITS2 rDNA regions were carried out on a MJ Research PTC-150 thermocycler (Waltham, MA). The following profile was used to amplify the LSU and ITS2 rDNA regions: an initial 96°C denaturation for 5 min, followed by 25 cycles of 96°C denaturation for 1 min, 54°C annealing for 15 s, 72°C extension for 30 s, and a final 72° Cextension for 4 min. The following profile was used to amplify the ITS1 region: an initial 95°C denaturation for 5 min, followed by 30 cycles of 95°C denaturation for 30 s, 55°C annealing for 30 s, 72°C extension for 1 min, and a final 72°C extension for 10 min. Amplified DNA was purified using QIAGEN® QIAquick™ PCR purification kit according to manufacturer's protocol. Cycle sequencing was conducted using the same primers utilized for PCR amplification with ABI Big Dye™ v.3.1 chemistry following manufacturer's recommendations. Precipitation with 3 M sodium acetate (pH approximately 5) and alcohol was done to remove dye terminators, and the pellets were then dried for 30–60 min at 39°C and sequenced using an ABI 3730xl automated sequencer. The resulting sequences were edited and contigs constructed using Sequencher™ version 4.5 (Gene Codes Corp.). GenBank accession numbers for all taxa sequenced in this study are provided below in the taxonomic summaries. The consensus sequences for each taxon utilized in this study were constructed from multiple replicates (each replicate being both a forward and reverse sequence from a single individual from different infections when possi-

ble) from different host/parasite/location combinations whenever possible.

Comparative DNA analyses

The entire ITS, 5.8S and partial LSU sequences generated for the species of *Varialvus* gen. nov. here were aligned initially to determine the amount of sequence variation among the various putative taxa. The LSU rDNA region for species of *Varialvus* was then aligned with data reported for species of the cryptogonimid genera *Adlardia* Miller, Bray, Goiran, Justine et Cribb, 2009; *Beluesca* Miller et Cribb, 2007; *Caulanus* Miller et Cribb, 2007; *Chelediadema* Miller et Cribb, 2007; *Latuterus* Miller et Cribb, 2007; *Lobosorchis* Miller et Cribb, 2005; *Neometadena* Hafeezullah et Siddiqi, 1970; *Retrovarium* Miller et Cribb, 2007 and *Siphoderina* Manter, 1934 *sensu* Miller and Cribb (2007a, c, d; 2008b) and Miller *et al*. (2009b) for comparative purposes and to explore levels of intra- and intergeneric variation. A second alignment dataset containing the ITS1, 5.8S and ITS2 rDNA regions for species of *Varialvus* and the cryptogonimid taxa above (excluding *Adlardia novaecaledoniae* Miller, Bray, Goiran, Justine et Cribb, 2009 because no ITS or 5.8S data was available) was also produced for comparative analysis. The ITS, 5.8S and LSU rDNA regions among species of *Varialvus* only and the other cryptogonimid taxa were initially aligned using ClustalX version 2.0.9 (Larkin *et al*. 2007) under the following parameters: pairwise alignment parameters = gap opening 10.00, gap extension 0.10, DNA weight matrix International Union of Biochemistry (IUB); and multiple alignment parameters = gap opening 10.00, gap extension 0.20, delay divergent sequences 30%, DNA weight matrix IUB. The resultant alignments were refined by eye using MacClade version 4.08 (Maddison and Maddison 2005). After the alignments of the ITS and LSU datasets were edited, the ends of each fragment were trimmed to match the shortest sequence in the alignment. Distance matrices among species of *Varialvus* gen. nov. over the ITS, 5.8S and LSU rDNA regions were constructed with the absolute and uncorrected 'p' pairwise character differences calculated using MESQUITE (Maddison and Maddison 2009) with gaps treated as a fifth unambiguous state.

Bayesian inference analysis of the LSU dataset was performed using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003) run on the CIPRES portal (Miller *et al.* 2009a) to explore relationships among these taxa. The software jModelTest version 0.1.1 (Guindon and Gascuel 2003, Posada 2008) was used to estimate the best nucleotide substitution model for the LSU dataset. Bayesian inference analysis was conducted on the LSU rDNA datasets using the GTR+I+G model predicted as the best estimator by the Akaike Information Criterion (AIC) in jModelTest. Bayesian inference analysis was run over 10,000,000 generations (ngen = 10000000) with two runs each containing four simultaneous Markov Chain Monte Carlo (MCMC) chains (nchains = 4) and every 1000th tree saved (samplefreq = 1000). Bayesian analyses used the following parameters: nst = 6, rates = invgamma, ngammacat = 4, and the MCMC parameters were left at the default settings, and the priors parameters of the combined dataset were set to ratepr = variable. Samples of substitution model parameters, and tree and branch lengths were summarised using the parameters 'sump burnin = 3000' and 'sumt burnin = 3000'. These 'burnin' parameters were chosen because the log likelihood scores 'plateaued' well before 3,000,000 replicates in the Bayesian inference analyses.

Results

Family Cryptogonimidae Ward, 1917

***Varialvus* gen. nov.**

Type-species: *Varialvus lacertus* sp. nov.

Diagnosis: Body fusiform to oval, longer than wide; length: width ratio 1:0.45–0.70. Tegument armed with small to minute spines. Eyespot pigment present in anterior half of body. Oral sucker nearly spherical to slightly funnel-shaped, with <35 enlarged oral spines, opens terminally. Ventral sucker unspecialized. Oral:ventral sucker width ratio *c*. 1:0.3–0.6. Forebody occupies *c*. 28–40% of body length. Prepharynx approximately as long as pharynx, may be slightly longer or shorter. Oesophagus short, approximately as long as pharynx, may be slightly longer or shorter. Intestinal bifurcation immediately anterior to or midway between ventral sucker and pharynx. Caeca blind, terminate close to posterior end of body. Testes two, opposite to slightly oblique, in mid-hindbody. Cirrus and cirrus-sac absent. Seminal vesicle saccular to tubulosaccular, between ovary and ventral sucker. Common genital pore immediately anterior to ventral sucker. Gonotyl absent or present as two distinctly muscular lobes or as stalk-like structure with crown of spines immediately anterior to or ventral to ventral sucker. Ovary consists of relatively condensed mass of deep lobes, medial, ventral to or immediately anterior and adjacent to testes. Laurer's canal present. Seminal receptacle saccular, sometimes relatively large, dorsal to ovary or between ovary and ventral sucker. Vitelline follicles in 2 lateral groups, mainly in forebody, extend anterior to intestinal bifurcation to midway between intestinal bifurcation and pharynx or to level of pharynx, sometimes extend to near anterior margin of ovary. Uterine coils restricted to hindbody, extend from posterior end of body to ventral sucker. Excretory vesicle Y-shaped; arms extend to pharynx; excretory pore terminal at posterior end of body.

Etymology: The name *Varialvus* is derived from the Latin *varius*, meaning different, various or changing and *alvus* meaning belly. It is proposed in recognition of the gonotyl of species in this genus, which may be absent or when present may vary. It is to be treated as masculine.

Differential diagnosis: *Varialvus* gen. nov. is distinguished from all other cryptogonimid genera by the combination of a

fusiform to oval body, the relatively small number of large oral spines, a median ovary which is relatively condensed and highly lobed, opposite to slightly oblique testes, uterine loops that are restricted to the hindbody and extend well posterior to the testes, and vitelline follicles that are mainly in the forebody but may extend from the anterior margin of the ovary to well anterior to the intestinal bifurcation. Of these characters the distribution of the vitelline follicles is the most immediately discriminating.

***Varialvus lacertus* sp. nov.** (Fig. 1)

Description: Based on 10 specimens. Body fusiform to oval, longer than wide, widest between ovary and ventral sucker, 711 (507–865) × 375 (276–449); length:width ratio 1:0.53 (1:0.47–0.59). Eyespot pigment present in anterior half of body. Oral sucker 111 (90–139) × 136 (110–160). Oral spines 25 (22–27), length 23 (17–29). Ventral sucker 49 (38–62) × 55 (40–67). Oral:ventral sucker width ratio 1:0.40 (1:0.36–0.46). Forebody occupies 32 (28–37)% of body length. Prepharynx 39 (32–51) long. Pharynx 51 (38–56) × 60 (51–70). Ventral sucker:pharynx width ratio 1:1.1 (1:1.0–1.3). Oesophagus 36 (29–51) long. Intestinal bifurcation between ventral sucker and pharynx. Intestinal caeca blind, 413 (285–490) long, terminate close to posterior end of body. Testes two, opposite, in mid-hindbody, 107 (62–160) × 114 (67–154). Seminal vesicle tubulosaccular, ventral to or dextral or sinistral to seminal receptacle, between ovary and ventral sucker. Gonotyl absent. Genital pore immediately anterior to ventral sucker. Ovary deeply lobed, ventral to or immediately anterior and adjacent to testes, 95 (62–118) × 142 (91–179). Laurer's canal present. Seminal receptacle saccular, dorsal to or immediately anterior to ovary. Vitelline follicles in two lateral groups, extend from slightly posterior to ventral sucker to pharynx. Uterine coils restricted to hindbody, extend from posterior end of body to ventral sucker. Eggs small, darkly tanned, 21 (17–25) × 9 (7–11). Excretory vesicle Y-shaped bifurcates dorsal to ovary; arms extend to pharynx, 517 (364–631) long, excretory pore terminal at posterior end of body.

Comments: *Varialvus lacertus* is immediately distinguished from all of its congeners by having a body that is <900 µm long, a pharynx that is distinctly wider or of equal width to ventral sucker and a body that is less than four times as wide as the oral sucker.

Type host: *Lutjanus quinquelineatus* (Bloch, 1790).

Additional host: *L. fulvus* (Forster, 1801).

Type locality: Lizard Island, Great Barrier Reef (14°40′S, 145°27′E), Queensland, Australia.

Site: Intestine and pyloric caeca.

Prevalence: 9 of 10 (90%) in *L. quinquelineatus*; 4 of 13 (31%) in *L. fulvus*.

Deposited specimens: Holotype QM (G231931), 9 paratypes QM (G231932–G231940).

Molecular sequence data: ITS, 6 replicates (3 from *L. quinquelineatus* and 3 from *L. fulvus*); LSU, 4 replicates (2 from *L. quinquelineatus* and 2 from *L. fulvus*).

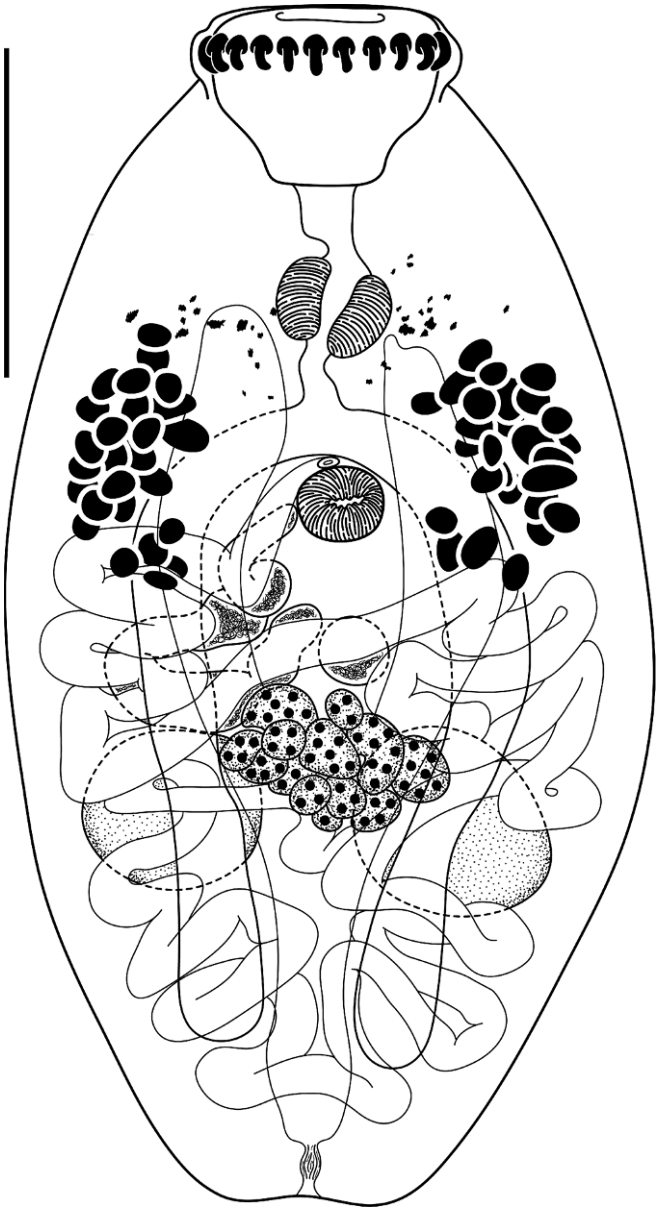

Fig. 1. *Varialvus lacertus* sp. nov. from the intestine of *Lutjanus quinquelineatus* off Lizard Island, Great Barrier Reef, Australia. Ventral view of holotype. Scale-bar = 200 µm

GenBank accession numbers: ITS (HM187780), LSU (HM187777).

Morphbank specimen number: (568322).

Etymology: The epithet *lacertus* is derived from the Latin *lacertus*, meaning lizard, referring to the type locality of this species. It stands as a noun in apposition.

***Varialvus charadrus* sp. nov.** (Fig. 2)

Description: Based on 28 specimens. Body fusiform to oval, longer than wide, widest between ovary and ventral sucker, 655 (410–852) × 357 (240–491); length:width ratio 1:0.55

(1:0.42–0.69). Eyespot pigment present in anterior half of body. Oral sucker 107 (74–153) × 122 (88–160). Oral spines 25 (22–28), length 21 (13–33). Ventral sucker 40 (26–58) × 42 (31–59). Oral:ventral sucker width ratio 1:0.34 (1:0.27–0.48). Forebody occupies 34 (29–38)% of body length. Prepharynx shorter than oesophagus, 19 (1–38) long. Pharynx 47 (32–65) × 52 (40–72). Ventral sucker:pharynx width ratio 1:1.3 (1:0.9–1.6). Oesophagus 36 (6–61) long. Intestinal bifurcation between ventral sucker and pharynx. Intestinal caeca blind, 396 (275–549) long, terminate close to posterior end of body. Testes two, opposite, in mid-hindbody, 155 (61–289) × 135 (70–210). Cirrus and cirrus-sac absent. Seminal vesicle tubulosaccular, between ovary and ventral sucker. Gonotyl present as two distinctly muscular lateral lobes immediately anterior to ventral sucker. Genital pore immediately anterior to ventral sucker. Ovary deeply lobed, ventral to or immediately anterior and adjacent to testes, 105 (42–157) × 137 (84–190). Laurer's canal present. Seminal receptacle saccular, between ovary and ventral sucker. Vitelline follicles in two lateral groups, extend from ventral sucker to pharynx. Uterine coils restricted to hindbody, extend from posterior end of body to ventral sucker. Eggs small, darkly tanned, 18 (15–20) × 8 (7–10). Excretory vesicle Y-shaped, bifurcates dorsal to ovary; arms extend to pharynx, 467 (293–644) long; excretory pore terminal at posterior end of body.

Comments: *Varialvus charadrus* is immediately distinguished from all of its congeners by having a gonotyl that lacks spines and consists of two distinctly muscular lateral lobes immediately anterior to the ventral sucker.

Type host: *Lutjanus vitta* (Quoy et Gaimard, 1824).

Additional hosts: *L. bohar* (Forsskål, 1775), *L. carponotatus* (Richardson, 1842), *L. fulviflamma* (Forsskål, 1775), *L. fulvus* (Forster, 1801), *L. gibbus* (Forsskål, 1775), *L. kasmira* (Forsskål, 1775), *L. quinquelineatus* (Bloch, 1790).

Type locality: Lizard Island, Great Barrier Reef (14°40′S, 145°27′E), Queensland, Australia.

Additional localities: Heron Island, Great Barrier Reef (23°26′S, 151°54′E), Queensland, Australia; Baie Maa, New Caledonia (22°13′S, 166°20′E); Reef near La Régnière, off Nouméa, New Caledonia (22°19′S, 166°18′E), Off Pointe Bovis, New Caledonia (22°14′S, 166°20′E) and; Rasdhoo Atoll (4°16′N, 72°58′E), Maldives.

Site: Intestine and pyloric caeca.

Prevalence: 4 of 11 (36%) in *L. vitta* at Lizard Island, 3 of 23 (13%) in *L. vitta* at New Caledonia; 1 of 11 (9%) in *L. bohar* at Lizard Island, 1 of 15 (7%) in *L. bohar* at Rasdhoo Atoll; 3 of 60 (5%) in *L. carponotatus* at Lizard Island, 1 of 144 (0.7%) in *L. carponotatus* at Heron Island; 3 of 36 (8%) in *L. fulviflamma* at Lizard Island, 4 of 13 (31%) in *L. fulvus* at Lizard Island; 3 of 7 (43%) in *L. gibbus* at Lizard Island, 4 of 5 (80%) in *L. gibbus* at Rasdhoo Atoll; 1 of 1 (100%) in *L. kasmira* at Lizard Island, 2 of 3 (67%) in *L. kasmira* at Rasdhoo Atoll; 2 of 3 (67%) in *L. quinquelineatus* at Heron Island, 6 of 10 (60%) in *L. quinquelineatus* at Lizard Island and 1 of 8 (12.5%) in *L. quinquelineatus* at New Caledonia.

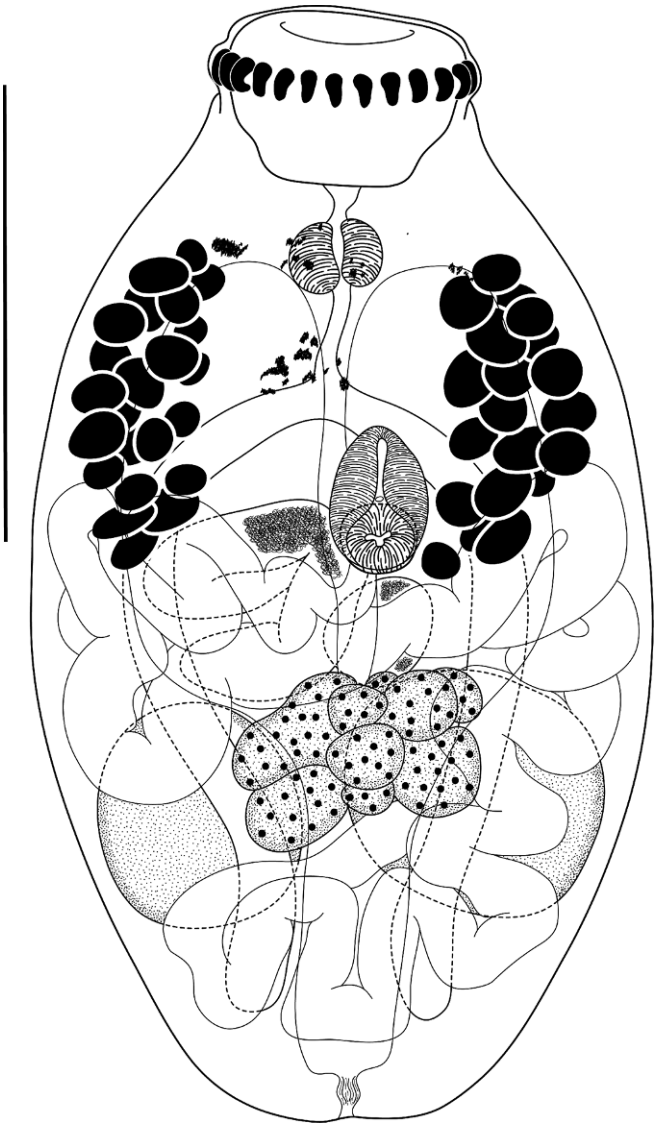

Fig. 2. *Varialvus charadrus* sp. nov. from the intestine of *Lutjanus vitta* off Lizard Island, Great Barrier Reef, Australia. Scale-bar = 200 μm

Deposited specimens: Holotype QM (G231941), 17 paratypes QM (G231942–G231958), 4 paratypes MNHN (JNC2143, 2655, 2688, 2689), BMNH 00000.

Molecular sequence data: ITS: 9 replicates from Heron Island (1 from *L. carponotatus* and 8 from *L. quinquelineatus*); 14 replicates from Lizard Island (4 from *L. vitta*, 1 from *L. bohar*, 1 from *L. carponotatus*, 1 from *L. fulviflamma*, 2 from *L. fulvus*, 3 from *L. gibbus*, 1 from *L. kasmira* and 1 from *L. quinquelineatus*); 10 replicates from Rasdhoo Atoll (4 from *L. bohar*, 4 from *L. gibbus* and 2 from *L. kasmira*). LSU: 5 replicates from Heron Island (1 from *L. carponotatus* and 4 from *L. quinquelineatus*); 4 replicates from Lizard Island (1 from *L. vitta*, 1 from *L. bohar*, 1 from *L. gibbus* and 1 from *L. quinquelineatus*); 4 replicates from Rasdhoo Atoll (2 from *L. bohar*, 1 from *L. gibbus* and 1 from *L. kasmira*).

GenBank accession numbers: ITS (HM187781), LSU (HM187778).

Morphbank specimen number: (568323).

Etymology: The epithet *charadrus* is derived from the Greek *charadra*, meaning deep gully or ravine, in recognition of the area between the lobes of the gonotyl of this species, which resembles a deep gully or valley.

***Varialvus jenae* sp. nov.** (Fig. 3)

Description: Based on 15 specimens. Body fusiform to oval, longer than wide, widest between ovary and ventral sucker, 638 (456–912) × 308 (205–488); length:width ratio 1:0.48 (1:0.45–0.54). Eyespot pigment present in anterior half of body. Oral sucker 93 (74–125) × 120 (99–167). Oral spines 28 (24–33), length 18 (14–23). Ventral sucker 55 (43–72) × 56 (43–80). Oral:ventral sucker width ratio 1:0.46 (1:0.42–0.53). Forebody occupies 37 (34–39)% of body length. Prepharynx 25 (13–35) long. Pharynx 40 (30–61) × 46 (37–62). Ventral sucker:pharynx width ratio 1:0.82 (1:0.74–0.93). Oesophagus 31 (14–45) long. Intestinal bifurcation between ventral sucker and pharynx. Intestinal caeca blind, 350 (250–528) long, terminate midway between posterior margin of testes or close to posterior end of body. Testes two, opposite to slightly oblique, in mid-hindbody, 81 (35–128) × 82 (51–122). Seminal vesicle tubulosaccular, dextral, ventral or sinistral to seminal receptacle, between ovary and ventral sucker. Gonotyl absent. Genital pore immediately anterior to ventral sucker. Ovary deeply lobed, ventral to or immediately anterior and adjacent to testes, 95 (62–138) × 143 (91–209). Laurer's canal present. Seminal receptacle saccular, between ovary and ventral sucker. Vitelline follicles in two lateral groups, extend from slightly posterior to ventral sucker to pharynx. Uterine coils restricted to hindbody, extend from posterior end of body to ventral sucker. Eggs small, darkly tanned, 16 (14–19) × 7 (7–8). Excretory vesicle Y-shaped, bifurcates dorsal to ovary; arms extend to pharynx, 500 (371–736) long; excretory pore terminal at posterior end of body.

Comments: *Varialvus jenae* is immediately distinguished from all of its congeners by having a pharynx that is narrower than the ventral sucker.

Type host: *Lutjanus carponotatus* (Richardson, 1842).

Type locality: Lizard Island, Great Barrier Reef (14°40′S, 145°27′E), Queensland, Australia.

Site: Intestine and pyloric caeca.

Prevalence: 23 of 60 (38%).

Deposited specimens: Holotype QM (G231959), 14 paratypes QM (G231960–G231973).

Molecular sequence data: ITS, 17 replicates; LSU, 4 replicates.

GenBank accession numbers: ITS (HM187779), LSU (HM187776).

Morphbank specimen number: (568324).

Etymology: The epithet *jenae* is in honour of Mrs Jennifer Miller in recognition of her support and encouragement of this work.

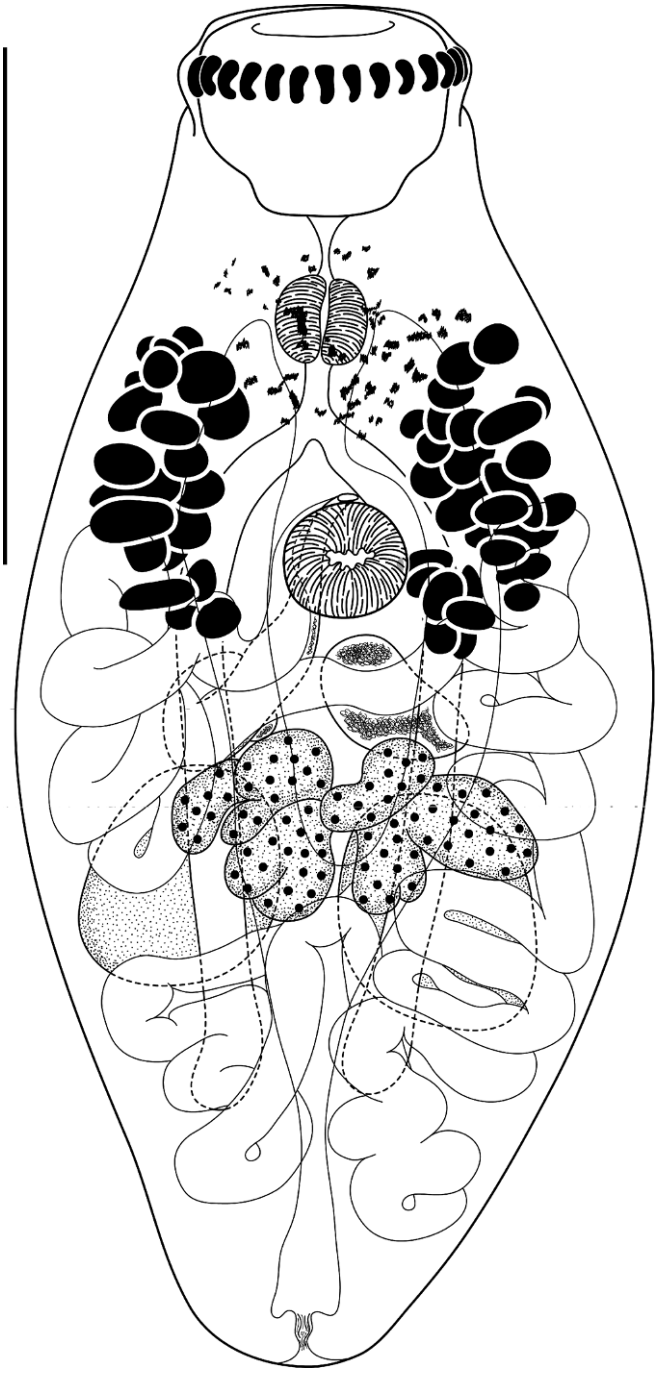

Fig. 3. *Varialvus jenae* sp. nov. from the intestine of *Lutjanus carponotatus* off Lizard Island, Great Barrier Reef, Australia. Scale-bar = 200 μm

***Varialvus angustus* sp. nov.** (Fig. 4)

Description: Based on 4 specimens. Body oval, longer than wide, widest between ovary and ventral sucker, 1,176 (928–1,304) × 584 (432–672); length:width ratio 1:0.49 (1:0.47–0.52). Eyespot pigment present in anterior half of body. Oral sucker 95 (82–99) × 121 (110–128). Oral spines 28 (26–29),

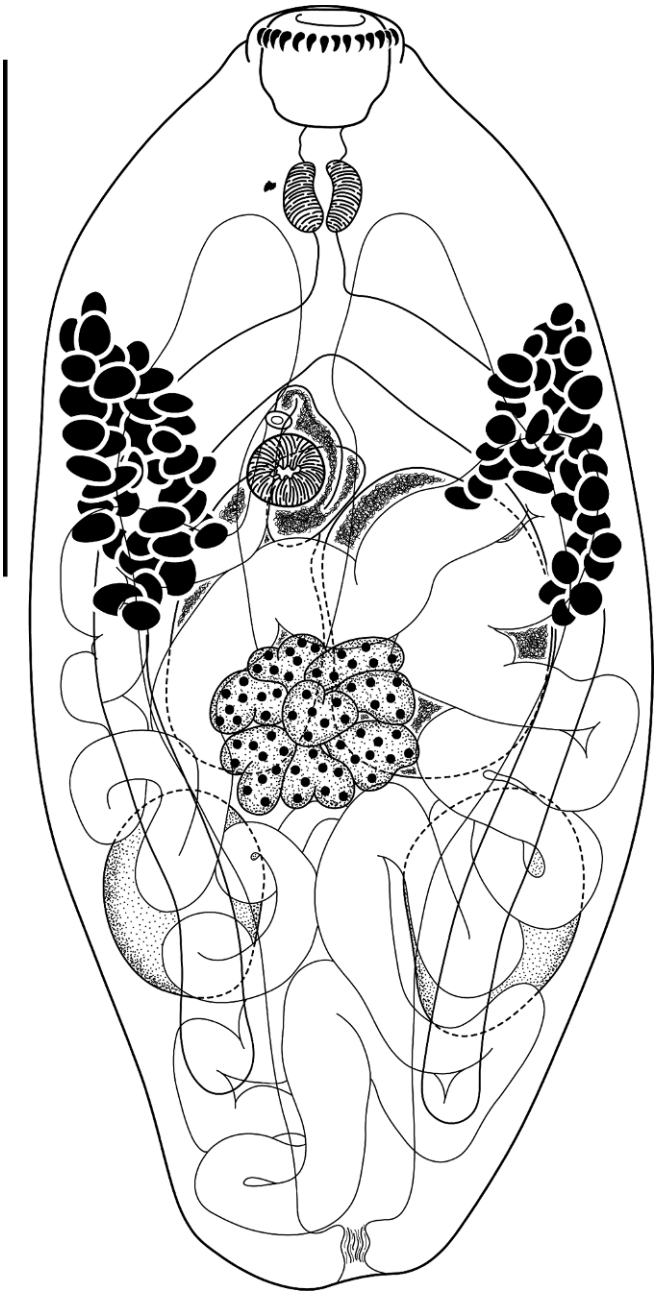

Fig. 4. *Varialvus angustus* sp. nov. from the intestine of *Lutjanus fulviflamma* off Lizard Island, Great Barrier Reef, Australia. Scale-bar = 500 µm

length 17 (13–21). Ventral sucker 70 (64–72) × 75 (69–78). Oral:ventral sucker width ratio 1:0.62 (1:0.59–0.63). Forebody occupies 34 (30–37)% of body length. Prepharynx shorter than oesophagus, 36 (32–42) long. Pharynx 63 (50–69) × 74 (67–77). Ventral sucker:pharynx width ratio 1:0.98 (1:0.96–1.02). Oesophagus 73 (67–83) long. Intestinal bifurcation midway between ventral sucker and pharynx. Intestinal caeca blind, 853 (631–975) long, terminate close to posterior end of body. Testes two, opposite to slightly oblique, in mid-hindbody, 207 (160–240) × 151 (118–189). Seminal vesicle saccular, dextral or sinistral to seminal receptacle, extends from dorsal to ovary to genital pore. Gonotyl absent. Genital pore immediately anterior to ventral sucker. Ovary deeply lobed, immediately anterior and adjacent to testes, 157 (93–192) × 154 (86–202). Laurer's canal present. Seminal receptacle saccular, extends from dorsal to ovary to ventral sucker. Vitelline follicles in two lateral groups, extend from near anterior margin of ovary to slightly posterior to pharynx. Uterine coils restricted to hindbody, extend from posterior end of body to ventral sucker. Eggs small, darkly tanned, 16 (15–18) × 8 (7–10). Excretory vesicle Y-shaped, bifurcates dorsal to ovary; arms extend to pharynx, 966 (752–1072) long; excretory pore terminal at posterior end of body.

Comments: *Varialvus angustus* is distinguished from all of its congeners by having a body that is approximately four times as wide or greater than the oral sucker, a ventral sucker that is greater than half as wide as the oral sucker (oral sucker:ventral sucker ratio 1:0.59–0.63) and of nearly equal width to the pharynx and a body length that is >925 µm.

Type host: *Lutjanus fulviflamma* (Forsskål, 1775).

Type locality: Lizard Island, Great Barrier Reef (14°40´S, 145°27´E), Queensland, Australia.

Site: Intestine and pyloric caeca.

Prevalence: 2 of 36 (6%).

Deposited specimens: Holotype QM (G231974), 3 paratypes QM (G231975–G231977).

Molecular sequence data: No molecular sequence data is available for this species.

Morphbank specimen number: (568325).

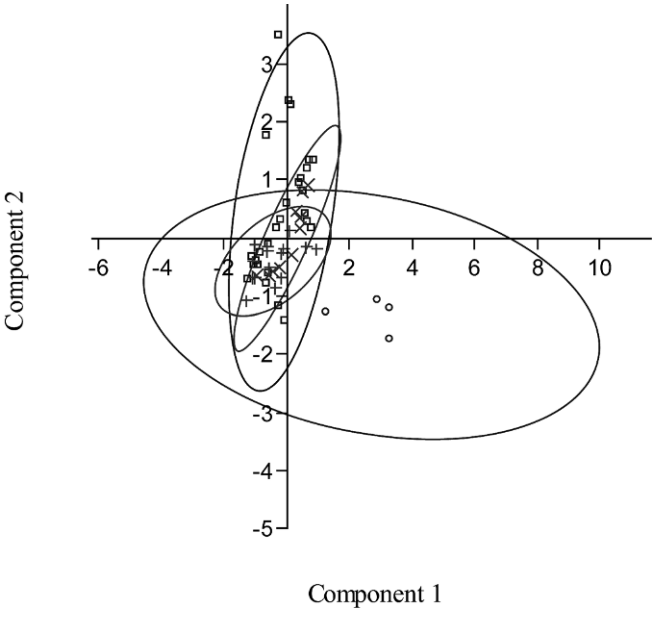

Fig. 5. Scatterplot indicating the positions of individuals of *Varialvus angustus* (circles), *V. charadrus* (squares), *V. jenae* (crosses) and *V. lacertus* (x's) based on Principal Components Analysis (PCA) of morphometric data. The 95% confidence circles are drawn for reference to show potential overlap

Etymology: The epithet *angustus* is derived from the Latin *angustus*, meaning narrow, referring to the narrow oral sucker width relative to body width in this species.

Principal components and discriminant analyses

Principal components analyses conducted on morphometric data between species of *Varialvus* (component axis 1, 88.4%, axis 2, 4.9%) resulted in a scatterplot that showed no distinct separation of *V. charadrus*, *V. jenae* and *V. lacertus* (Fig. 5). *Varialvus angustus* was the only taxon that displayed statistically significant differences in morphology, with all four individuals grouping separate to the other three taxa along the component axes (Fig. 5).

Discriminant analysis conducted on the morphometric data for species of *Varialvus* indicated that the oral sucker to ventral sucker ratio (F-to-remove = 11.3) was the character that most contributed to the statistically significant variation observed between these taxa. The next most significant character distinguishing these two taxa in discriminant analysis was body length (F-to-remove = 4.76).

Key to species of *Varialvus* gen. nov.

1a. Muscular gonotyl present 2
1b. Muscular gonotyl absent 3
2a. Gonotyl stalk-like, with crown of spines .... *V. lutjani* (Saoud, Ramadan et Al Kawari, 1988) n. comb.
2b. Gonotyl consists of two distinctly muscular lobes..... ..... *V. charadrus* sp. nov.
3a. Body width approximately four times as wide or greater than the oral sucker width; body length >925 µm .... *V. angustus* sp. nov.
3b. Body width less than four times as wide as oral sucker width; body length <925 µm 4
4a. Pharynx distinctly wider or of equal width to ventral sucker ..... *V. lacertus* sp. nov.
4b. Pharynx narrower than ventral sucker .... *V. jenae* sp. nov.

Comparative DNA analysis

ITS and 5.8S rDNA

Alignment of the entire ITS (ITS1 and ITS2) and 5.8S rDNA region for the species of *Varialvus* for which molecular data was available yielded 1,087 characters (base pairs and gaps) for analysis. The ITS1 region varied in length between these species; 569 bp in *V. lacertus*, 570 bp in *V. charadrus* and 573 bp in *V. jenae*. The 5.8S region was 157 bp in length in all species of *Varialvus* examined here. The ITS2 rDNA region was 288 bp in both *V. lacertus* and *V. charadrus*, but was 294 bp in *V. jenae*. The number of base pair differences and percentage of uncorrected 'p' distances between species of *Varialvus* over the ITS1, 5.8S and ITS2 regions are shown in Table I. No intraspecific variation was observed in any of the taxa over the ITS rDNA region.

Variation in the 5' half of the ITS1 made alignment in this region between species of *Varialvus* and all of the other cryptogonimid taxa examined here impossible, so only the 3' half of the ITS1 was included for comparative purposes as in Miller and Cribb (2007a, c, d), because this region was easily aligned. Alignment of the ITS dataset containing the other cryptogonimid taxa, which included the 3' end of the ITS1, the entire 5.8S and ITS2 and 11 bp of the 5' end of the LSU yielded 878 characters for analysis. Over this dataset, species

Table I. Genetic variation among species of *Varialvus* gen. nov. over the internal transcribed spacers (ITS1 and ITS2), 5.8S and partial large subunit (LSU) rDNA regions. Values to the left of the diagonal represent the absolute number of base pair differences and those in parentheses to the right of the diagonal the percentage of uncorrected 'p' pairwise distance. The total number of replicates for each of the rDNA regions sequenced for each taxa are listed under the column labelled 'n'

	n	*V. lacertus*	*V. charadrus*	*V. jenae*
ITS1				
Varialvus lacertus	6	–	**(15.3)**	**(6.8)**
Varialvus charadrus	33	**73**	–	**(13.9)**
Varialvus jenae	17	**33**	**71**	–
5.8S				
Varialvus lacertus	6	–	**(0.6)**	**(0.6)**
Varialvus charadrus	33	**1**	–	**(1.3)**
Varialvus jenae	17	**1**	**2**	–
ITS2				
Varialvus lacertus	6	–	**(14.9)**	**(8.8)**
Varialvus charadrus	33	**41**	–	**(16.2)**
Varialvus jenae	17	**18**	**38**	–
LSU				
Varialvus lacertus	4	–	**(4.8)**	**(2.3)**
Varialvus charadrus	13	**41**	–	**(4.6)**
Varialvus jenae	4	**19**	**38**	–

of *Varialvus* differed in uncorrected 'p' distance from species of *Beluesca* by 7.5–11.7%, *Caulanus* by 9.4–12.4%, *Chelediadema* by 15–17.2%, *Gynichthys* by 13.1–15.2%, *Latuterus* by 8.5–12.5%, *Lobosorchis* by 11.4–16.1%, *Neometadena* by 16.4–16.5%, *Retrovarium* by 12–18.2% and *Siphoderina* by 8.2–13.7%.

LSU rDNA

Alignment of the LSU rDNA region for species of *Varialvus* gen. nov. and the remainder of the cryptogonimid taxa examined yielded 861 characters for analysis. The number of base pair differences between species of *Varialvus* over the partial LSU region are shown in Table I. No intraspecific variation was observed in any of the *Varialvus* species sequenced over the LSU rDNA region. Over this dataset, species of *Varialvus* differed in uncorrected 'p' distance from species of *Adlardia* by 7.8–8.6%, *Beluesca* by 4.3–6.7%, *Caulanus* by 5.1–6.4%, *Chelediadema* by 10.2–11.2%, *Gynichthys* by 8.2–8.5%, *Latuterus* by 6.1–8.2%, *Lobosorchis* by 8.6–9%, *Neometadena* by 7.5–8.4%, *Retrovarium* by 6.5–9.3% and *Siphoderina* by 5.1–8.1%.

Bayesian inference analysis of the LSU rDNA dataset resulted in a phylogram in which species of *Varialvus* forming a strongly resolved clade with species of *Beluesca* (Fig. 6). The clade containing species of *Varialvus*, *Beluesca*, *Caulanus*, *Latuterus* and *Siphoderina* was poorly resolved and sister to a well-resolved clade containing the remainder of the taxa examined. All genera were resolved with high posterior probability support in the Bayesian Inference analysis performed here.

Discussion

Systematics

Species of *Varialvus* gen. nov. most closely resemble those of the genera *Allacanthochasmus* Van Cleave, 1922, *Anoiktostoma* Stossich, 1899, *Neochasmus* Van Cleave et Mueller, 1932, *Parspina* Pearse, 1920 and *Siphoderina* Manter, 1934. Species of *Anoiktostoma* differ from those of *Varialvus* in having a uterus that extends well into the forebody to near the pharynx. Species of *Allacanthochasmus* differ from those of *Varialvus* in having an ovary that forms a distinctly transverse band that occupies nearly the entire body width and they are apparently restricted to freshwater fishes (Moronidae). Species of *Neochasmus* are distinguished from those of *Varialvus* in having a gonotyl that is present as muscular pad, sucker-like structure or non-muscular lobes immediately posterior to the common genital pore and vitelline follicles that are restricted to the hindbody. The 'thin-walled pouch' enclosing the seminal vesicle, small pars prostatica and short ejaculatory duct and apparent restriction to freshwater Siluriformes distinguishes species of *Parspina* from those of *Varialvus*. Species of *Varialvus* can be distinguished from those of *Siphoderina* (which also infect lutjanids and haemulids) by the combination of having relatively few large oral spines, vitelline follicles that are confined mainly to the forebody and extend to the level of or well anterior to the intestinal bifurcation.

Ribosomal DNA sequence data was obtained for *V. charadrus*, *V. jenae* and *V. lacertus* (unfortunately no DNA data was obtained for *V. angustus*) and confirmed the close relationships between these species suggested by morphology (Fig. 6). The intra- and intergeneric genetic distances in the ITS, 5.8S and LSU rDNA regions observed for species of *Varialvus* here were also similar to that seen for other cryptogonimid taxa (Miller and Cribb 2007a, c, d, 2008a, 2009; Miller *et al*. 2009a, b). In the absence of DNA sequence data, the distinctly muscular gonotyl of *V. charadrus* might have warranted placement within a separate genus (along with *V. lutjani* n. comb., see below), but rDNA sequence data confirms its placement within *Varialvus*. A similar situation was seen with the cryptogonimid *Retrovarium planum* Miller et Cribb, 2007 (Miller and Cribb 2007a), whose distinctive morphology would have also warranted placement in a novel genus without rDNA data. The use of DNA sequence data here to elucidate the systematic relationships among these species of Cryptogonimidae has proven effective in creating biologically accurate taxonomic classifications within the family.

Allacanthochasmus lutjani Saoud, Ramadan et Al Kawari, 1988 was proposed by Saoud *et al*. (1988) for specimens recovered from *Lutjanus fulviflamma* from the Arabian Gulf off Qatar. This species was tentatively transferred to *Siphoderina* by Miller and Cribb (2008b) because it did not agree with species of *Allacanthochasmus*, which are restricted to freshwater teleosts from North America. This species agrees with *Varialvus* gen. nov. in that it has a relatively small number of large oral spines, the vitelline follicles are confined mainly to the forebody, a gonotyl is present and its host is a species of *Lutjanus*. Based on these similarities it is here transferred as *V. lutjani* (Saoud, Ramadan et Al Kawari, 1988) n. comb. The structure of the gonotyl of *V. lutjani* was not fully characterized or figured properly by Saoud *et al*. (1988), but the SEM micrograph and the brief description provided suggest the presence a crown of spines encircling a stalk-like gonotyl, which in combination with its relatively large size and vitelline follicles that do not extend posterior to the ventral sucker, distinguishes it from the remaining species of *Varialvus*.

Biogeographical and host distribution

At least one species of the genus *Varialvus* appears to be widely distributed in the Indo-West Pacific, similar to other recently reported cryptogonimid taxa (Miller and Cribb 2007a, c; Miller *et al*. 2009a). All of the new species of *Varialvus* reported here were recovered only from Lizard Island, except for *V. charadrus*, which was found at Lizard and Heron Islands on the Great Barrier Reef, New Caledonia and the Maldives; identical rDNA (ITS, 5.8S and LSU) sequences obtained

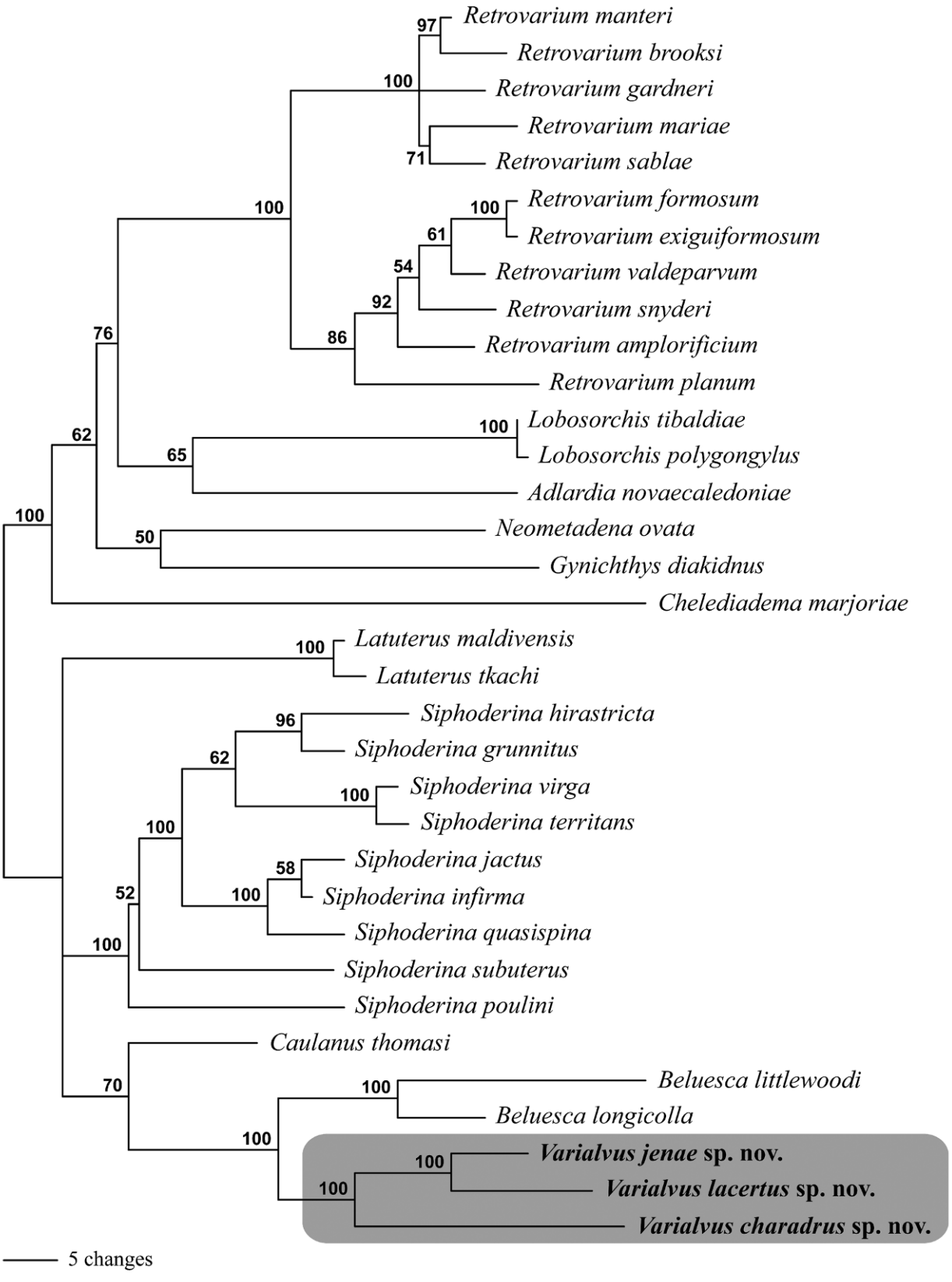

Fig. 6. Relationships between species of *Varialvus* gen. nov. and the remainder of the cryptogonimid taxa examined here based on Bayesian inference analysis of the LSU rDNA dataset. Posterior probabilities are shown at the nodes with values <50 not shown. The phylogram is mid-point rooted

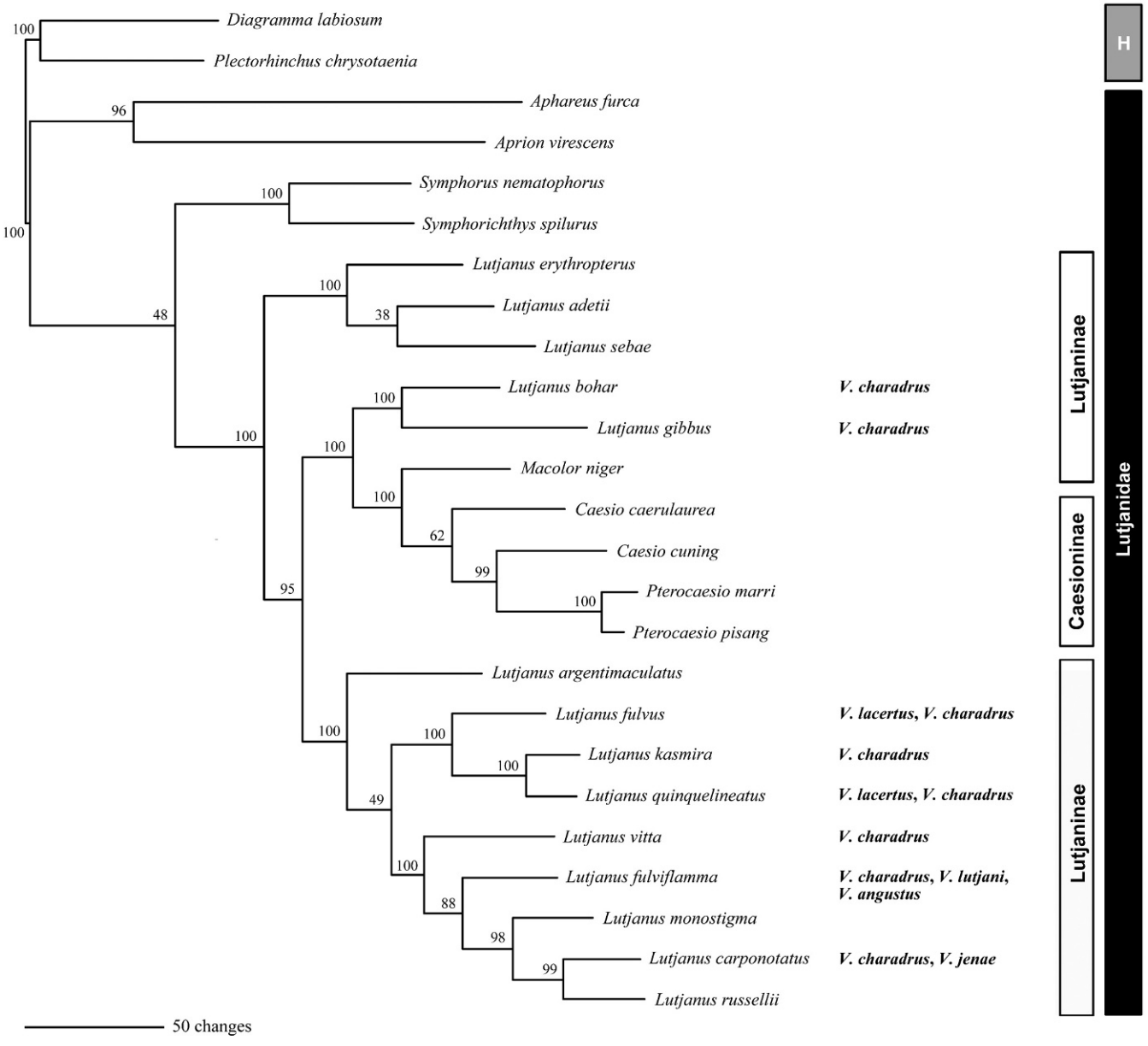

Fig. 7. Host distribution of species of *Varialvus* gen. nov. reported here mapped onto the phylogeny of Indo-Pacific Lutjanidae produced by Miller and Cribb (2007a, b)

from specimens from the Great Barrier Reef and the Maldives (no molecular samples were available from New Caledonia) confirm that it is a widely distributed species. Morphological and molecular data confirm that this species has a biogeographic range of at least 10,000 kilometres in the Indo-West Pacific. *Varialvus jenae* was observed at 38% (23 of 60 hosts examined) prevalence in *L. carponotatus* at Lizard Island, but in none of the 144 individuals examined at Heron Island, strongly suggesting that it does not occur there. Further study is needed to determine why some species of *Varialvus* are widespread while others exhibit more restricted biogeographic distributions.

Host specificity and distribution varied widely among the species of *Varialvus*. Two species, *V. jenae* and *V. angustus* were recovered from only a single host species each (*L. carponotatus* and *L. fulviflamma*, respectively), *V. lacertus* was recovered from two species, *L. quinquelineatus* and *L. vitta*, and *V. charadrus* was found in an exceptional eight host species. None of the species infecting multiple fish species had host distributions of lutjanids that were each other's closest relatives (Fig. 7). The distribution of *V. charadrus* would lead to the prediction that *L. monostigma* and *L. russellii* are likely to be infected with that species at Heron or Lizard Islands, but more individuals of these lutjanids need to be ex-

amined at these localities in order to confirm this. Interestingly, *V. charadrus* was found in two major lineages of lutjanines that were examined in this survey, one containing *L. bohar* and *L. gibbus* and the other containing the 'blackspot' and 'blue-lined' clades (see Allen 1985, Miller and Cribb 2007b) as well as the species *L. argentimaculatus*, *L. fulvus* and *L. vitta*. Identical rDNA sequences obtained from this broad range of hosts in addition to the prevalences observed provide evidence supporting this broad, yet disjunct host distribution.

Acknowledgements. This study was funded by, and is a contribution from, the Australian node of the CReefs global research initiative (grant number: 209/29), a partnership between the Australian Biological Resource Study (ABRS), BHP Billiton, the Great Barrier Reef Foundation, the Census of Marine Life and the Australian Institute of Marine Science (AIMS). The CReefs Australia Project is generously sponsored by BHP Billiton in partnership with The Great Barrier Reef Foundation, the Australian Institute of Marine Science, the Australian Biological Resources Study and the Alfred P. Sloan Foundation. CReefs is a field program of the Census of Marine Life. We thank Dr Matthew Nolan, Mieke Burger, and Dr Nicole Gunter for assistance with the fieldwork undertaken on the Great Barrier Reef and for help in collecting the teleost specimens used in this study. We gratefully thank the staff of the Lizard Island research station for their support and hospitality. We also thank Dr Robert Konecny and Julia Lorber for helping to organize the trip to the Maldives and for their assistance in the field. We are grateful to the Research Service and International Relations Office of the University of Vienna for assistance in financing the trip to Rasdhoo Atoll, Maldives. Dr Reinhard Kikinger kindly gave us access to his laboratory at the Kuramathi Biological Field Station to conduct fish necropsies and extended his generous hospitality to us during our stay. In New Caledonia, Dr František Moravec, Eva Řehulkova, Jean-Pierre Debenay, Bernard Marchand and several students including Aude Sigura, Cyndie Dupoux and Marine Briand participated to the fishing expeditions and parasitological survey.

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(Accepted July 11, 2010)