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Interrelationships of the Haploporinae (Digenea: Haploporidae): A molecular test of the taxonomic framework based on morphology

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ABSTRACT

The taxonomic framework of the Haploporidae is evaluated and the relationships within the Haploporinae are assessed for the first time at the generic level using molecular data. Partial 28S and complete ITS2 rDNA sequences from representatives of six of the nine recognised genera within the Haploporinae were analysed together with published sequences representing members of two haploporid subfamilies and of the closely related family Atractotrematidae. Molecular analyses revealed: (i) a close relationship between the Atractotrematidae and the Haploporidae; (ii) strong support for the monophyly of the Haploporinae, *Dicrogaster* and *Saccocoelium*, and the position of *Ragaia* within the Haploporinae; (iii) evidence for rejection of the synonymy of *Saccocoelioides* and *Lecithobotrys* and the validity of the Dicrogasterinae; and (iv) support for the distinct status of *Saccocoelium* in relation to *Haploporus*. The wider sampling within the genera *Dicrogaster* and *Saccocoelium* confirmed the distinct status of the included species, thus rejecting previously suggested synonymies. *Saccocoelioides*, recently transferred to the Chalcinotrematinae, was nested within the Haploporinae and this was largely associated with the position of *Forticulcita*, resolved as the most basal haploporine genus. *Forticulcita* also possesses a well-delimited eversible intromittent copulatory organ, a feature unique in the Haploporidae which has not been previously considered an important apomorphy. This, in association with the present hypothesis of the Haploporinae based on molecular data, led us to erect Forticulcitinae subf. n. for *Forticulcita*; this resolved *Saccocoelioides* and, by extension the Chalcinotrematinae, as sister groups to the Haploporinae.

1. Introduction

The Haploporinae Nicoll, 1914, one of the four currently recognised supragenerics taxa within the Haploporidae Nicoll, 1914 [1], is a group of poorly known digeneans which parasitise marine or brackish water mugilid fishes (Mugilidae). Looss [2] erected the majority of its genera (*i.e. Haploporus* Looss, 1902, *Dicrogaster* Looss, 1902, *Lecithobotrys* Looss, 1902 and *Saccocoelium* Looss, 1902) for a few species which he described from Mediterranean mullets. His descriptions and generic diagnoses were brief and based on a small number of specimens; this has resulted in subsequent misleading identifications and synonymies leading to an underestimation of the diversity of Mediterranean haploporines. Thus, Dawes [3] considered *Haploporus lateralis* Looss, 1902 a synonym of *H. benedeni* Looss, 1902; Dawes [3] supported by Mikailov [4], Fischthal and Kuntz [5], Ferretti and Paggi [6] and Moravec and Libosvárský [7] regarded *Saccocoelium obesum* Looss, 1902 and *S. tensum* Looss, 1902 synonymous; and Dawes [3] and Sarabeev and Balbuena [8] synonymised *Dicrogaster contracta* Looss, 1902 with *D. perpusilla* Looss, 1902.

The problems in haploporine taxonomy extend to generic recognition as well. Thus *Saccocoelioides* Szidat, 1954, originally assigned to the Haploporinae by Szidat [9], was considered a subgenus of *Lecithobotrys* by Yamaguti [10] (later reinstated, see [11]) and a junior synonym of *Lecithobotrys* by Nasir and Gómez [12]. Overstreet and Curran [1] temporarily accepted the validity of *Lecithobotrys*, reorganised *Saccocoelioides* and transferred *Saccocoelioides* (*sensu stricto*) to the new subfamily Chalcinotrematinae Overstreet & Curran, 2005. These authors also suggested that *Lecithobotrys* may be synonymous with *Haploporus* and indicated that the placement of some species of *Haploporus* and *Saccocoelium* is difficult. Yamaguti [10] erected the Dicrogasterinae Yamaguti, 1958 for *Dicrogaster* based on the presence of a single vitellarium (*vs.* vitellarium in two symmetrical masses in his concept of the Haploporinae [10,11]) but this action was not accepted by Overstreet and Curran [1].

In spite of the large number of records of haploporine species especially in Mediterranean mullets, there are surprisingly few documented records (*i.e.* supplied with a description or figure) or taxonomic studies contributing to the knowledge of morphological

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variation in this group (see [13–15] for detailed lists). In a recent revision of the Mediterranean genera of the Haloporidae six of the species (*i.e. Haloporus benedeni*, *Dicrogaster contracta*, *D. perpusilla*, *Lecithobotrys putrescens* Looss, 1902, *Saccocoelium obesum* and *S. tensum*) originally described by Looss [2] were redescribed from newly collected material, three new species (*Saccocoelium cephali* Blasco-Costa et al., 2009, *S. currani* Blasco-Costa et al., 2009 and *Forticulcita gibsoni* Blasco-Costa et al., 2009) were described, and a new genus was erected for a new species, *Ragaia lizae* Blasco-Costa et al., 2009, from the Ebro Delta. The status of the species previously assigned to the four genera erected by Looss [2] was examined and keys to generic and species level were developed [13–16]. Simultaneously, we sequenced the second internal ribosomal spacer (ITS2) region and the partial large subunit rRNA (28S) gene of haloporine representatives of all Mediterranean haloporine genera.

In this study we evaluate the taxonomic framework of the Haloporinae based on morphology [1,13–16] using ribosomal DNA sequence data generated from 10 species representing six out of the nine genera currently recognised within the subfamily. We test the monophyly of the subfamily by incorporation of the only available sequence data for two non-haloporine haloporids and two atractotrematid species, and assess for the first time relationships at the generic level. More specifically, the molecular data allowed an independent test of the previous hypotheses for the synonymy of *Lecithobotrys* and *Saccocoelioides* as well as for the status of *Haloporus*, *Lecithobotrys* and *Saccocoelium*. In two cases the data provided an opportunity to test earlier suggested synonymies at the species level.

2. Materials and methods

2.1. Taxon sampling

Specimens representing all Mediterranean haloporid genera were collected from *Mugil cephalus* L., *Liza aurata* (Risso), *L. ramado* (Risso) and *L. saliens* (Risso) at three localities along the Mediterranean coast of Spain [Ebro Delta (40°30′–40°50′N, 0°30′–1°10′E), off Santa Pola (38°00′–38°20′N, 0°10′–0°40′E) and in a brackishwater lagoon near Santa Pola]. In total, 34 sequence replicates of both ITS2 and partial 28S rDNA regions of 10 species were obtained (see Table 1 for hosts, localities and sequence/specimen accession numbers). Multiple replicate sequences (*i.e.* obtained from different individual worms) for the two gene regions of six species [*Dicrogaster perpusilla* (5 specimens); *D. contracta* (7 specimens); *Haloporus benedeni* (4 specimens); *Lecithobotrys putrescens* (2 specimens); *Saccocoelium obesum* (4 specimens); and *S. tensum* (7 specimens)] were obtained in order to test for intraspecific variability. All haloporid taxa sequenced are described morphologically in Blasco-Costa et al. [13–16]. Type- and voucher material has been deposited in the British Museum (Natural History) Collection at the Natural History Museum, London (BMNH) and sequences were submitted to GenBank (Table 1).

2.2. DNA extraction, amplification and sequencing

Specimens fixed live in 100% EtOH and stored at −20 °C were subsequently transferred into 300 µl TNES urea extraction buffer [10 mM Tris–HCl (pH 8), 125 mM NaCl, 10 mM EDTA, 0.5% SDS, 4 M urea]. Genomic DNA was extracted from single specimens using a phenol-chloroform protocol described previously [17]. Alternatively, 1 M Tris–EDTA (pH 8) buffer was used to replace ethanol from the tissue of some specimens and gDNA was extracted using Qiagen® DNeasy™ tissue kit following manufacturer's protocol, except for the proteinase-K incubation period, which was extended overnight, and the gDNA was further concentrated to a volume of ~30 µl using Millipore Microcon® columns. Complete ITS2 rDNA sequences were amplified using primers 3S (5 GTA CCG GTG GAT CAC GTG GCT AGT G-3′) [18] and ITS2.2 (5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′) [18]. Partial (domains D1–D3; ~1400 bps) 28S rDNA sequences were amplified using primers LSU5 (5′-TAG GTC GAC CCG CTG AAY TTA AGC A-3′) [19] and LSU1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) [20] or U178 (5′-GCA CCC GCT GAA YTT AAG-3′) [21] and L1642 (5′-CCA GCG CCA TCC ATT TTC A-3′) [21]. Polymerase chain

Table 1
List of the taxa incorporated in the molecular analyses (new sequences indicated by a star) with host, locality, sequence (EMBL/GenBank) and specimen (BMNH[a]) accession data.

Taxon	Host	Locality	GenBank accession numbers		BMNH accession numbers
Subfamily Haloporinae			28S	ITS2	
Ingroup taxa					
Forticulcita gibsoni	*M. cephalus*	Santa Pola (sea)[b]	FJ211239*	FJ211249*	2008.10.7.61–76
Ragaia lizae	*L. aurata*	Ebro Delta[b]	FJ211235*	FJ211245*	2008.10.7.19
Lecithobotrys putrescens	*L. saliens*	Ebro Delta[b]	FJ211236*	FJ211246*	2008.10.7.56–60
Haloporus benedeni	*L. ramado*	Santa Pola (sea)[b]	FJ211237*	FJ211247*	2008.10.7.52–55
Dicrogaster perpusilla	*L. ramado*	Santa Pola (lagoon)[b]	FJ211238*	FJ211248*	2008.10.7.6–11
Dicrogaster contracta	*L. aurata*/*L. ramado*	Santa Pola (sea)[b]	FJ211261*/FJ211262*	FJ211267*/FJ211268*	2008.10.7.12–13/ 2008.10.7.14–16
Saccocoelium n. sp.	*L. saliens*	Ebro Delta[b]	FJ211234*	FJ211244*	2008.10.7.82–83
Saccocoelium cephali	*M. cephalus*	Ebro Delta[b]	FJ211233*	FJ211243*	2008.10.7.23–25
Saccocoelium obesum	*L. aurata*/*L. ramado*	Ebro Delta[b]	FJ211260*/FJ211259*	FJ211266*/FJ211265	2008.10.7.38–39
Saccocoelium tensum	*L. aurata*/*L. ramado*	Ebro Delta[b] Santa Pola (lagoon)[b] Santa Pola (sea)[b]	FJ211258*/FJ211257*	FJ211264*/FJ211263*	2008.10.7.41–43/ 2008.10.7.44
Subfamily Chalcinotrematinae					
Saccocoelioides sp.	Unidentified molly (Poecilidae)	Nicaragua	EF032696	–	–
Subfamily Megasoleninae					
Hapladena nasonis	*Naso unicornis*	Lizard Island, Australia	AY222265	–	–
Family Atractotrematidae					
Pseudomegasolena ishigakiense	*Scarus rivulatus*	Heron Island, Australia	AY22266	–	–
Atractotrema sigani	*Siganus lineatus*	Lizard Island, Australia	AY222267	–	–
Outgroup taxa					
Paragonimidae					
Paragonimus westermani	'experimentally infected final host'	Meghlaya, India	DQ836244	DQ836243	–
Lepocreadiidae					
Preptetos trulla	*Ocyurus chrysurus*	Kingston, Jamaica	AY222237	–	1995.9.26.1–5
Preptetos laguncula	*Naso unicornis*	Heron Island, Australia	–	AF392439	–

[a] BMNH, British Museum (Natural History) Collection at the Natural History Museum, London.
[b] Spain.

reaction (PCR) amplifications were carried out using Ready-To-Go™ (Amersham Pharmacia Biotech) PCR beads (each containing ~1.5 units *Taq* DNA polymerase, 10 mM Tris–HCl at pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP and stabilisers, including BSA), 20–70 ng of template DNA and 10 mM of each PCR primer. The following thermocycling profile was used for ITS2 rDNA amplification: denaturation of DNA (95 °C for 3 min); 35 cycles of amplification (94 °C for 50 s, 54 °C for 50 s and 72 °C for 1 min 20 s); and 4 min extension hold at 72 °C. The same profile but with annealing temperatures of 58° and 56 °C respectively, for the primer combinations LSU5–L1500R and U178–L1642, was applied for 28S rDNA amplification. PCR amplicons were either gel-excised or purified directly using Qiagen QIAquick™ PCR Purification Kit and cycle-sequenced from both strands using ABI BigDye™ Terminator v3.1 Ready Sequencing Kit, alcohol-precipitated, and run on an ABI 3730 automated sequencer. The PCR primers and internal primers 300F (5′-CAA GTA CCG TGA GGG AAA GTT G-3′), ECD2 (5′-CTT GGT CCG TGT TTC AAG ACG GG-3′) and LSU1200R (5′-GCA TAG TTC ACC ATC TTT CGG-3′) [19] in the case of the 28S rDNA products, were used for cycle sequencing. Contiguous sequences were assembled and edited using either Bioedit v7.0.5. (©1997–2005 [22]) or Sequencher™ (GeneCodes Corp. ver. 3.1.1.).

2.3. Alignments and phylogenetic analysis

The new ITS2 rDNA and partial 28S rDNA sequences were aligned in two independent datasets, the latter including the chalcinotrematine haploporid *Saccocoelioides* sp., the megasolenine haploporid *Hapladena nasonis* Yamaguti, 1970, and two species (*Atractotrema sigani* Durio and Manter, 1969 and *Pseudomegasolena ishigakiensis* Machida and Kamiya, 1976) representing the closely related family Atractotrematidae Yamaguti, 1939 [see 23] for which sequences were available for this region only. Species of *Paragonimus* Braun, 1899 (Paragonimidae) and *Preptetos* Pritchard, 1960 (Lepocreadiidae) were chosen to root the phylogenetic trees (see Table 1 for details). Sequences were aligned initially using ClustalX [24] with default parameter values and adjustments made by eye using MacClade ver. 4.08 [25]. Regions of ambiguous alignment, characterised by the presence of indels of varying length, were defined in a character exclusion set and thus removed from the analyses.

The two datasets were analysed individually *via* Bayesian inference (BI) and maximum parsimony (MP). BI analyses were conducted using MrBayes ver. 3.1.2 [26] and the nucleotide substitution models were estimated independently for each dataset using ModelTest ver. 3.06 [27]. The best fitting model was the general time reversible with estimates of invariant sites and gamma-distributed among-site rate variation (GTR + I + Γ) in the case of 28S, and the general time reversible model with an estimate of gamma-distributed among-site rate variation (GTR + Γ) in the case of ITS2. For BI, two simultaneous, independent analyses with four chains each (default behaviour in ver. 3.1.2) were run for 1 million generations with default priors and a sample frequency of 100, and tested for convergence of their posterior probability distributions. 'Burn-in' was estimated by plotting log-likelihoods against generation and determining when these values and substitution parameters had plateaued. This was generation 12,300 and 30,000 for the 28S and ITS2 regions, respectively. Nodal support was estimated as posterior probabilities [28]. MP analyses were performed with PAUP* ver. 4.0b10 [29] using a heuristic search strategy with 1000 search replicates, random-addition taxon sampling, tree-bisection-reconnection branch-swapping, with all characters run unordered with equal weights and with gaps treated as missing data. Nodal support was estimated by bootstrap analysis (heuristic search strategy with 1000 pseudoreplicates and 100 random sequence addition each). Distance matrices (percentage of pairwise character differences with gaps treated as missing data) for each rDNA region were calculated with PAUP* after trimming the ends of the data sets to match the shortest sequences.

3. Results

A total of 337 and 1029 included (*i.e.* alignable) characters were available for analysis in the ITS2 and 28S datasets, respectively. Of these, 179 (53%) and 649 (63%) were invariant, and 84 (25%) and 232 (23%) informative under the principles of parsimony, respectively. No intraspecific variability was found in all cases where sequences were obtained from multiple representatives of a species. Within the Haploporinae (*sensu* Overstreet and Curran [1] and Blasco-Costa et al. [15]), the interspecific sequence variability ranged from 2.1 to 10.9% in ITS2 and from 0.9 to 4.8% in 28S (*Dicrogaster* spp. and *Saccocoelium* spp., see Table 2). Intergeneric divergence showed a slight overlap with the species-level data ranging between 6.7–21.2% and 4.6–11.4%, respectively. The upper limits of intergeneric divergence within the Haploporinae were set by *Forticulcita* whereas the pairwise comparison of *Lecithobotrys* and *Haploporus* showed the lowest percent of sequence divergence (Table 2). Comparisons at the suprageneric level (28S dataset only) largely overlapped with the intergeneric data for haploporine subfamilies (range of 9.1–14.5%) and were slightly higher 12.3–15.8% for family level comparisons (see Table 2 for details).

The data available for two representatives of other haploporid subfamilies (*i.e.* the Megasoleninae Manter, 1935 and the Chalcinotrematinae) and two species of the Atractotrematidae, the latter considered the closest [1,30] or synonymous with the Haploporidae [31], allowed their inclusion in the analyses of the 28S sequences. Fig. 1 presents a species-level phylogram of the Haploporidae based on Bayesian inference of the 28S rDNA dataset. The tree topologies

Table 2
Pairwise nucleotide sequence comparisons between taxa for the aligned 28S rDNA sequences ($N = 1029$ nt) (below the diagonal) and for the aligned ITS2 rDNA sequences ($N = 337$ nt) (above the diagonal).

	Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Saccocoelium cephali*	–	5.1	10.9	10.9	9.4	10.3	9.6	11.2	10.2	21.1				
2	*Saccocoelium tensum*	2.6	–	10.2	9.0	8.2	10.0	9.6	10.5	9.6	19.7				
3	*Saccocoelium obesum*	4.6	3.8	–	2.1	14.1	14.4	13.6	13.7	13.3	21.1				
4	*Saccocoelium* n. sp.	4.8	4.2	0.9	–	13.2	14.7	13.9	13.1	12.4	21.2				
5	*Ragaia lizae*	6.3	5.8	5.8	6.4	–	8.8	8.8	11.3	10.7	19.4				
6	*Lecithobotrys putrescens*	7.5	7.2	7.6	7.9	6.4	–	6.7	11.0	11.0	21.0				
7	*Haploporus benedeni*	8.6	7.9	8.5	8.9	6.8	4.6	–	10.6	9.4	19.3				
8	*Dicrogaster perpusilla*	8.2	7.6	8.1	8.5	6.8	8.1	8.2	–	8.7	20.6				
9	*Dicrogaster contracta*	6.6	5.8	6.2	6.6	5.2	6.5	6.8	4.6	–	17.3				
10	*Forticulcita gibsoni*	10.5	10.5	10.5	11.0	10.7	10.9	10.7	11.4	10.2	–				
11	*Saccocoelioides* sp.	9.8	9.6	9.9	10.2	9.7	10.6	11.2	11.0	9.4	9.1	–			
12	*Pseudomegasolena ishigakiense*	13.1	12.4	12.9	13.3	13.2	13.9	13.6	14.6	13.5	12.7	12.3	–		
13	*Atractotrema sigani*	15.1	14.0	14.0	14.3	15.3	15.2	14.6	15.8	14.9	14.8	14.3	12.0	–	
14	*Hapladena nasonis*	13.5	13.0	12.9	13.0	13.7	14.2	13.1	14.5	13.3	13.9	13.5	13.2	15.8	–

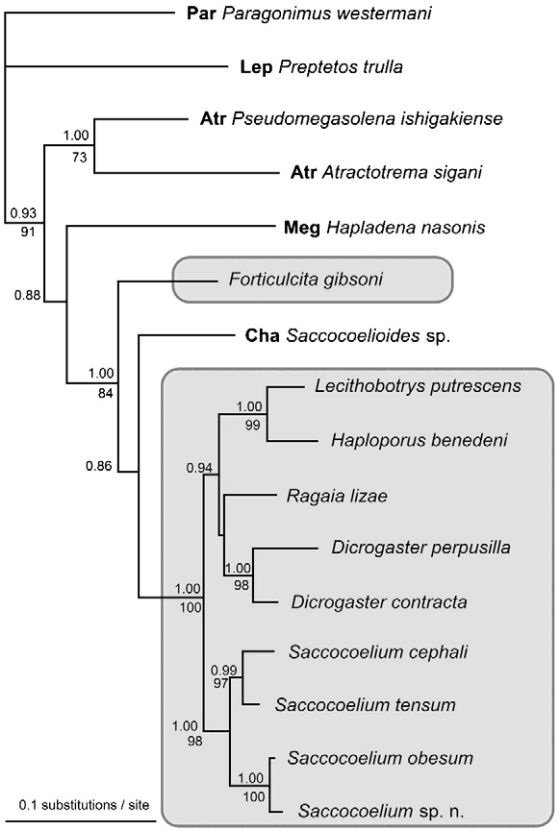

Fig. 1. Bayesian inference phylogram employing a GTR + I + Γ substitution model for 28S rDNA dataset. Nodal support is provided by posterior probabilities (number above or only number) and by maximum parsimony bootstrap percentages (number below; not shown if <70%). Shaded areas indicate the Haploporinae *sensu* Overstreet and Curran [1]. *Abbreviations*: Atr, Atractotrematidae; Cha, Chalcinotrematinae; Lep, Lepocreadiidae; Meg, Megasoleninae; Par, Paragonimidae.

generated with MP and BI analyses of the 28S dataset were both congruent and highly supported. The Haploporinae formed a strongly supported monophyletic clade, with *Saccocoelioides* sp. nested within it. Within this clade, *Forticulcita* occupied a basal position, sister to *Saccocoelioides* and the rest of the haploporines which formed two clades with high support: one including *Saccocoelium* spp. and the other including *Dicrogaster* spp. together with *Ragaia*, *Lecithobotrys*, and *Haloporus*. The placement of *Ragaia* was unclear due to its weakly supported position in the individual trees of both datasets, appearing either as sister taxon to *Dicrogaster* (28S, see Fig. 1) or to *Saccocoelium* (ITS2, see Fig. 2). The topology depicted by the analyses of the ITS2 dataset was poorly supported for most nodes, except for the *Saccocoelium* spp. clades, and the Haploporinae (however excluding *Forticulcita*) which had high posterior probabilities and bootstrap values (Fig. 2). Regarding suprageneric relationships (28S dataset only), in both analyses *Hapladena* appeared basal in the Haploporidae, but poorly supported, whereas the Atractotrematidae formed a strongly supported sister clade to the Haploporidae.

4. Discussion

The systematic position and taxonomy of the family Haploporidae still offer challenges at several levels. Thus, the poor knowledge of the morphological and molecular diversity within this family has resulted in contradictory (morphological data [32]) or unclear (molecular data [23]) concepts for the placement of the family Haploporidae within the classification scheme of the Digenea. The recent taxonomic revision of Overstreet and Curran [1] has greatly clarified the situation at the generic/suprageneric level. Molecular systematic studies aimed at these taxonomic levels have generally produced the most conclusive results in a number of digenean families [33].

This first attempt, using independent molecular data, at assessing the interrelationships of the Haploporidae provides a test of the taxonomic framework based on morphology; the wider sampling within the family was aimed at improving the knowledge of the relationships at higher taxonomic levels. The results resolve, with considerable resolution, the relationships among and within the genera of the Haploporidae available for analysis. Although not directly comparable, analyses of the 28S rDNA dataset provided stronger support than those based on the ITS2 dataset, including robust support for a clade representing the Mediterranean genera of the Haploporinae. Analyses of the ITS2 dataset resulted in a topology showing some differences to 28S; these were, however, poorly supported due to a higher degree of homoplasy in the ITS2 leading to inaccurate phylogenetic inference [34,35]. Although sequences in the present ITS2 dataset did not exhibit length variation posing difficulties in alignment (discussed in Nolan and Cribb [35]) we base our discussion of haploporid interrelationships primarily on the results from 28S, but consider both gene regions with regard to estimating pairwise distances among the taxa.

4.1. Comparisons of genetic distance

Relatively low values of intergeneric variation (*i.e.* 4.6–8.6% and 6.7–14.7% for the 28S and ITS2, respectively) were observed within the Haploporinae (excluding *Forticulcita*, see below). These fall within the range observed among the genera of the Cryptogonimidae (28S: 3.8–8.4%; ITS2: 6.6–12% [36,37]) and Didymozoidae (ITS2: 3.0–19.0 [38]) and well below that reported for the Bivesiculidae (ITS2: 16.0 to 36.0% [39]). The intergeneric divergence appears closely associated with the interspecific sequence variation recorded in the latter three examples for which data are available at both levels, *i.e.* very low in

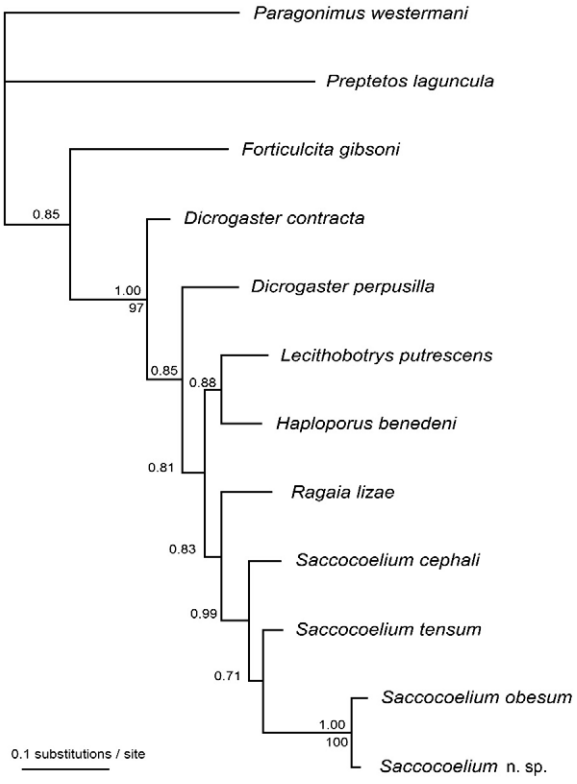

Fig. 2. Bayesian inference phylogram employing a GTR + Γ substitution model for ITS2 rDNA dataset. Nodal support is provided by posterior probabilities (number above or only number) and by maximum parsimony bootstrap percentages (number below; not shown if <70%).

Cryptogonimidae (0.2–0.4% in the 28S and 0.4–7.1% in the ITS2) and Didymozoidae (0.5% in the ITS2) *vs.* 8.1–11.6% in the ITS2 of *Bivesicula* [see 36–39]. Therefore, the lower limits of genetic differentiation observed in our study at the species level (0.9% and 2.1% in the 28S and ITS2, respectively) may not be exceptional and may have implications for species recognition within the family Haploporidae (*i.e.* sibling species are likely to be found within other haploporid lineages).

4.2. Subfamily-level interrelationships

Skrjabin [40] placed *Dicrogaster* within the Haploporinae, whereas Yamaguti [10] erected the Dicrogasterinae Yamaguti, 1958 for this genus. Overstreet and Curran [1] did not consider the Dicrogasterinae to be valid, and their conclusion is well-supported by our molecular analyses that place *Dicrogaster* within the Haploporinae as sister to *Haploporus* (type-genus) and *Lecithobotrys* (Fig. 1). The two species of *Dicrogaster* differed considerably in terms of the two rDNA regions and this confirms their distinct species status, in agreement with our recent morphological study [14].

Haploporus, the type-genus of the Haploporinae, was strongly associated with *Lecithobotrys* (Fig. 1). Further, the type-species of both genera exhibited the lowest percentage of sequence difference, which falls within the interspecific range observed within *Saccocoelium* and *Dicrogaster*. These results tend to support the possible synonymy suggested by Overstreet and Curran [1], although sequence data from more species of both genera are needed to adequately circumscribe their limits before a nomenclatural change can be recommended. Currently we distinguish morphologically *Lecithobotrys* from *Haploporus* based on: (i) the shape and size of the seminal vesicles (both elongate-oval and external distinctly larger than internal *vs.* subglobular and similar in size); (ii) the genital atrium (distinct, with muscular wall *vs.* shallow and lacking muscular wall); and (iii) the structure of the vitellarium (in two separated lateral clusters of distinct subglobular groups of small coalesced follicles *vs.* two separated compact masses) [13]. Altogether then, there appears to be a considerable disjunction between morphology and molecules in the case of *Lecithobotrys* and *Haploporus*.

The present phylogenetic hypotheses did not resolve the closest affinities of *Ragaia*, recently erected for *R. lizae* from the Mediterranean, although its inclusion among the haploporines was well-supported. It is possible that its sister genus has not yet been described since only three recognised haploporine genera are not included in the present analyses due to lack of data (*Unisaccus* and *Pseudodicrogaster* from the Indo-West Pacific and the poorly defined *Rondotrema* parasiting non-mugilid fishes in the Southwest Atlantic). The largely disparate geographical distribution of these genera, however, makes the possibility of a close relationship with *Ragaia* seem unlikely.

Saccocoelium formed a strongly supported monophyletic group. The wider sampling within this genus allowed confirmation of the distinct status of *S. tensum* and *S. obesum* (thus rejecting previously suggested synonymy, see Introduction) and the recently described *S. cephali* plus one new species of *Saccocoelium* (Figs. 1 and 2). We found no evidence to question the distinct status of *Saccocoelium* (especially in relation to *Haploporus*) as the two genera clustered in different, well-defined clades. A characteristic feature of *Saccocoelium* that distinguishes it morphologically from *Haploporus* and all known genera of the Haploporidae is the presence of a prominent genital atrium with a strongly developed muscular wall [15,16]. Therefore, the problems [1] with species affiliation to either genus are due to poor differential diagnoses and incorrect taxonomic assignments rather than to morphological similarity resulting from shared inheritance.

4.3. Phylogenetic inference at the familial level

Our results clearly resolve the distinct status of *Saccocoelioides*, recently transferred to the subfamily Chalcinotrematinae by Overstreet and Curran [1], as well as that of *Lecithobotrys*, thus rejecting the synonymy suggested by Yamaguti [10] and Nasir and Gómez [12]. The two genera were found clustering in different well-supported groups, *Saccocoelioides* being earlier divergent to the clade including *Lecithobotrys*. However, *Saccocoelioides* was found to be nested within the Haploporinae (*sensu* Overstreet and Curran [1]) and this is largely associated with the position of *Forticulcita* which was resolved as the most basal haploporine genus (Fig. 1); *F. gibsoni* also exhibited the highest percentage of sequence difference in the pairwise comparisons with all other species of the Haploporinae.

Arising from the present phylogenetic solutions two alternatives can be suggested. If the current classification of Overstreet and Curran [1] is considered, *i.e. Forticulcita* as basal within the Haploporinae, the position of *Saccocoelioides* sp. would result in paraphyly of the Chalcinotrematinae, which was previously suggested by Overstreet and Curran [1] when they erected the subfamily. *Saccocoelioides* was included in Chalcinotrematinae based on vitelline follicles surrounding the testis, the presence of a short oesophagus and an uterine loop anterior to the ventral sucker, and the developed miracidia having pigmented eye-spots [1]. However, the vitellarium in *Saccocoelioides* is not as well-developed as in the other genera of the Chalcinotrematinae and the vitelline follicles are arranged in two symmetrical groups rather than irregularly dispersed in lateral fields in the hindbody. Whereas the presence of eye-spots depends on the development of the miracidia [1], the structure of the vitellarium in *Saccocoelioides* suggests a closer resemblance to Haploporinae than Chalcinotrematinae. Definitely, additional sequences of identified species (preferably the type-species) of *Saccocoelioides* and other chalcinotrematines are required to test whether they form a natural group. Our results lead us to think that (i) *Saccocoelioides* may belong to the Haploporinae, or (ii) that *Saccocoelioides*, and by extension the Chalcinotrematinae, is the closest group to the Haploporinae.

On the other hand, *Forticulcita* possesses diagnostic morphological features that appear to be unique/rare in the Haploporidae: a single vitelline mass (present only in *Dicrogaster fastigata* Thatcher & Sparks, 1958) and an eversible ejaculatory organ (terminology of Overstreet [41]). This muscular structure (long and cylindrical when everted [14]) is present in all three species of the genus, *i.e. Forticulcita glabra* Overstreet, 1982, *F. gibsoni* Blasco-Costa et al., 2009, and *F. mugilis* Hassanine, 2007; described as eversible hermaphroditic duct for the latter [14,41,42]. However, the presence of an intromittent ejaculatory organ has not been previously considered an important apomorphy; this feature, although originally included in the generic diagnosis of *Forticulcita* by Overstreet [41], was not considered in the generic, subfamilial or familial diagnoses in the recent revision of the Haploporidae [1]. Although the ability of the hermaphroditic duct to evert has been described in some haploporids [e.g. 13,14,16,43,44], we consider the presence of a well-delimited eversible intromittent copulatory organ an important discriminating feature at the subfamilial level. This, combined with the present hypothesis of the Haploporinae inferred from rDNA sequence data, suggests that taxonomic elevation of *Forticulcita* is warranted; the latter resolves *Saccocoelioides* and the Chalcinotrematinae as sister group to the Haploporinae. We therefore erect the subfamily Forticulcitinae for the latter with the following diagnosis:

Forticulcitinae subfam. n.

Haploporidae. Body fusiform, with maximum width at level of ventral sucker. Tegument armed. Eye-spot pigment dispersed between oral sucker and hermaphroditic sac. Oral sucker subterminal. Ventral sucker about size of oral sucker or larger. Forebody short. Prepharynx short. Pharynx large, subspherical. Oesophagus 2–6 times length of pharynx. Caeca two, sac-like, end blindly at about mid-body or more posterior. Testis single, dextral to submedian. External seminal vesicle tubular, distinctly longer than internal seminal vesicle. Hermaphroditic sac elongate, subcylindrical. Internal seminal vesicle tubular to elongate-oval. Hermaphroditic duct narrow. Ejaculatory organ

muscular, cylindrical. Genital atrium shallow. Genital pore median, just anterior to ventral sucker. Ovary pretesticular, contiguous with or overlapping testis. Metraterm long. Eggs numerous, operculate; developed miracidia with single or two fused eye-spots. Vitellarium a single large spherical to subtriangular compact mass of small follicles, at level of or posterior to gonads. Excretory system Y-shaped, pore terminal, wide. Type-genus: *Forticulcita* Overstreet, 1982.

Although *Hapladena* appeared as the most basal taxon in the Haloporidae, the relationships of the sole species of the subfamily Megasoleninae for which sequence is currently available, *H. nasonis*, remained unresolved; its position was also found to be labile, as sister to either the Atractotrematidae or the Haloporidae, in analyses including a much wider taxonomic sampling (Blasco-Costa et al., unpublished results). This species was found to form a strongly supported clade with the Atractotrematidae in an analysis of the relationships of the Acanthocolpidae Lühe, 1906 [45], a sister taxon to the Haloporidae [23]. On the other hand, *H. nasonis* grouped as a sister taxon to the newly sequenced chalcinotrematine haloporid, *Saccocoelioides* sp. in a study on a different set of taxa closely related to the Haloporidae by Curran et al. [30]; the poor support of the latter relationship was interpreted as evidence for a distant relationship between the two subfamilies. However, these authors have excluded from the analysis the sequence of the second atractotrematid species (*i.e. Atractotrema sigani*) and this might have affected the topology of the tree. Although our results sustain the assumption of a distant relationship between the Megasoleninae and the Haloporinae also supported by host-parasite data (all members of the former subfamily occur in marine reef fishes and none was found in a mugilid [1]), it is unfortunate that only a single taxon of the most speciose megasolenine genus has so far been used in all molecularly tested hypotheses. However, *H. nasonis* appears to be an aberrant ('atypically elongate' [see 1]) representative of the Megasoleninae. Clearly, the relationships of this subfamily would be better understood if sequences of type-taxa were incorporated in future analyses.

Finally, there was strong support for a close relationship between the Atractotrematidae, currently recognised as a distinct family within the Haloporoidea Nicoll, 1914 [1,46], and the Haloporidae. Wider sampling within the Atractotrematidae and of the remaining subfamilies of the Haloporidae would improve our knowledge of their relationships so that natural groups could be better defined and the validity of the Atractotrematidae assessed.

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