Host specificity versus plasticity: testing the morphology-based taxonomy of the endoparasitic copepod family Splanchnotrophidae with COI barcoding

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The Splanchnotrophidae is a family of highly modified endoparasitic copepods known to infest nudibranch or sacoglossan sea slug hosts. Most splanchnotrophid species appear to be specific to a single host, but some were reported from up to nine different host species. However, splanchnotrophid taxonomy thus far is based on external morphology, and taxonomic descriptions are, mostly, old and lack detail. They are usually based on few specimens, with intraspecific variability rarely reported. The present study used molecular data for the first time to test (1) the current taxonomic hypotheses, (2) the apparently strict host specificity of the genus Ismaila *and (3) the low host specificity of the genus* Splanchnotrophus *with regard to the potential presence of cryptic species. Phylogenetic analyses herein used sequences of the barcoding region of the cytochrome oxidase I (COI) gene from 40 specimens representing 13 species of five genera. Species delimitation approaches include distance and barcoding gap analyses, haplotype networks and diagnostic nucleotides. Molecular results are largely compatible with the commonly accepted, morphology-based taxonomy of the Splanchnotrophidae. Strict host specificity could be confirmed for two* Ismaila *species. COI analyses also supported the idea that* Splanchnotrophus angulatus *is host-promiscuous. In* Ismaila, *morphology seems more suitable than barcoding to display speciation events via host switches in a recent Chilean radiation. In* Splanchnotrophus, *some genetic structure suggests ongoing diversification, which should be investigated further given the inadequate morphology-based taxonomy. The present study thus supports the presence of two different life history strategies in splanchnotrophids, which should be explored integratively.*

Keywords: species delimitation, molecular phylogeny, DNA taxonomy, speciation, Copepoda, sea slugs, parasite

INTRODUCTION

Copepods are the most abundant and speciose group in marine habitats (Yoshikoshi, 1975; Ho, 2001; Blanco-Berical *et al.*, 2014) and they also display the greatest variety of forms (Gotto, 1979, 2004; Ho, 2001; Blanco-Berical *et al.*, 2014). Endoparasitic copepods often exhibit extremely aberrant body forms due to the high level of adaptation to their respective host (Gotto, 1979, 2004; Huys, 2001; Haumayr & Schrödl, 2003; Anton *et al.*, 2015). Such is the case in Splanchnotrophidae Hancock & Norman, 1863, a family of bizarre endoparasitic copepods exclusively infesting nudibranch and sacoglossan hosts. The family is distributed worldwide in temperate and warm coastal waters and currently

comprises six genera: *Splanchnotrophus* Hancock & Norman, 1863, *Ismaila* Bergh, 1867, *Lomanoticola* Scott & Scott, 1895, *Arthurius* Huys, 2001, *Ceratosomicola* Huys, 2001 and *Majimun* Uyeno & Nagasawa, 2012, with a total of now 32 species (Anton *et al.*, 2015). All members are characterized by an enhanced body size in females, the possession of dorsal appendages (with one exception, see Anton *et al.*, 2015), the reduction of the maxillipeds, and the abdomen of females protruding through the host's integument (Huys, 2001; Anton & Schrödl, 2013a, b).

The taxonomy of Splanchnotrophidae is exclusively based on external morphology, with descriptions offering a highly heterogeneous level of detail and reliability. In addition, the use of external morphological characters in highly modified endoparasitic taxa has to be regarded as problematic at best (Huys, 2001). In such a case, the differentiation between true homoplasies and convergent evolution is rather complex. Most splanchnotrophids (i.e. 25 species; 78%) are

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considered to be highly host specific, and usually each host species is infested by a single parasite species (Schrödl, 1997, 2003; Huys, 2001; Haumayr & Schrödl, 2003; Anton & Schrödl, 2013a, b); identification of an infested host thus may permit identification of their parasite. Interestingly, all members of the species-rich and recently reviewed genera *Ismaila* and *Ceratosomicola* are strictly host specific. A recent radiation of Chilean *Ismaila* species via host shifts was proposed (Schrödl, 2003; Anton & Schrödl, 2013a, b). However, some splanchnotrophids are reported from multiple hosts (Figure 1A). The recently revised or described genera *Arthurius* and *Majimun* (Huys, 2001; Salmen *et al.*, 2008; Uyeno & Nagasawa, 2012) comprise a few species that are host specific and others that infest multiple host species. Similarly, five of nine species of the taxonomically obscure genera *Splanchnotrophus* and *Lomanoticola* are reported from more than one (i.e. up to nine) different species (Figure 1A) of not necessarily closely related sea slug groups (Anton & Schrödl, 2013a). For example, *Lomanoticola brevipes* (Hancock & Norman, 1863) was reported infesting members of the dexiarchian nudibranch family Dotidae, but was also found in representatives of the aeolid families Flabellinidae, Tergipedidae, Facelinidae and Eubranchidae. Different splanchnotrophid genera and species thus display different patterns of host specificity, possibly reflecting phylogenetic constraints on their ability to detect, colonize or survive in different hosts (Anton & Schrödl, 2013a, b). It is also striking that five of the seven splanchnotrophids known from more than one host species occur exclusively in the Mediterranean Sea and along the European coasts of the Atlantic ocean (Figure 1B). These areas are among the earliest and most intensely studied with regard to marine invertebrates. However, neither the parasites nor their hosts are of apparent commercial value, and original or subsequent descriptions of European splanchnotrophids are typically old and usually based on single individuals with no adequate vouchers deposited for later study (Canu, 1891; Hecht, 1895; Bassett-Smith, 1903; O'Donoghue, 1924; Delamare Deboutteville, 1950).

Estimates of host specificity in splanchnotrophid copepods, and conclusions on the presence, ecology and evolution of highly heterogeneous specificity in different genera and geographic areas entirely depend on taxonomic identifications of parasites and hosts. On the host side, taxonomy appears straightforward, although the existence of cryptic species has only been tested by molecular data for two complexes. Both the *Cratena peregrina* (Gmelin, 1791) (Padula *et al.*, 2014) and the *Spurilla neapolitana* (Delle Chiaje, 1841) (Carmona *et al.*, 2014) complexes were split up using integrative taxonomic evidence. To date, splanchnotrophid taxonomy is exclusively based on (external) morphology, and little is known about intrapopulational variation (Anton & Schrödl, 2013a, b); taxonomically relevant features such as special details of mouth parts are unknown for several species, i.e. several but not all of the species described to inhabit different hosts (Huys, 2001; Haumayr & Schrödl, 2003; Anton & Schrödl, 2013a, b). In general, the morphology of endoparasites can be especially adapted to their environment, i.e. conditions in their hosts (Gotto, 1979; Huys, 2001). For example, large-sized hosts may allow for longer body lengths, and the morphology of the host may affect the position of the parasites inside the hosts. Therefore, it is a crucial task to evaluate phenotypic splanchnotrophid taxonomy using genetic data,

testing the assumption of narrowly adapted parasite species against host-induced plasticity. Anton & Schrödl (2013a, b) provided a morphocladistic hypothesis on the phylogeny of splanchnotrophids and also proposed a preliminary scenario of character evolution and coevolution of splanchnotrophids with certain host groups. Since parts of the tree were not robustly supported, investigating historic and recent coevolution requires molecular analyses. DNA sequence data for splanchnotrophids has been lacking entirely, due to the difficulty of collecting and preserving a variety of rare or at least sporadic endoparasites.

The present study for the first time uses molecular data to (1) test the current taxonomic hypotheses on Splanchnotrophidae introduced by Huys (2001) and recently confirmed by morphocladistic analysis (Anton & Schrödl, 2013a, b); (2) test the strict host specificity reported for the genus *Ismaila* (potentially leading to the highest species diversity of all splanchnotrophid genera) against undiscovered host-induced phenotypic plasticity; and (3) evaluate the supposedly low host specificity of *Splanchnotrophus* against the possibility of the presence of cryptic species.

To test general taxonomic hypotheses, phylogenetic analyses were conducted, using 38 novel barcode sequences of the cytochrome oxidase I (COI) gene from 12 morphospecies, covering four splanchnotrophid genera. To further study host specificity, species delimitation analyses were performed focusing on two supposedly strictly host-specific species of *Ismaila* (*Ismaila aliena* Haumayr & Schrödl, 2003, *Ismaila robusta* Haumayr & Schrödl, 2003) and on *Splanchnotrophus angulatus* Hecht, 1893, a species currently known from five different host species. Here, a variety of molecular methods complement and extend the traditional view on species boundaries in splanchnotrophids, and allows for a preliminary integrative view on life history traits such as host specificity.

MATERIALS AND METHODS

Species sampling

For molecular analyses all ethanol-fixed splanchnotrophid samples available in the collection of the Bavarian State Collection of Zoology (ZSM) were used to obtain genetic material. Additional samples of *I. aliena, I. robusta* and *S. angulatus* were gathered during several collection trips to southern Chile in 2008 and 2010, and to southern France in 2010. Wherever possible, egg sacs were carefully removed from the host using forceps as soon as possible after collection. Samples were then stored in 96% ethanol and kept chilled until the DNA extraction was performed. A detailed list of all included specimens is given in Table 1.

DNA extraction, amplification and sequencing

We used a NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) and extraction procedures followed manufacturers' instructions. Universal primers LCO-1490 (forward) and HCO-2198 (reverse) (Folmer *et al.*, 1994) were used to amplify a ~650 bp segment of the cytochrome oxidase I (COI) gene. For amplification Illustra PuRe Taq Ready-To-Go PCR beads (GE Healthcare) were used. A mix of 0.5 μl of each primer (conc. 10 pm, Metabion) plus 23 μl of molecular water was added to 1.0 μl of raw DNA. For PCR conditions
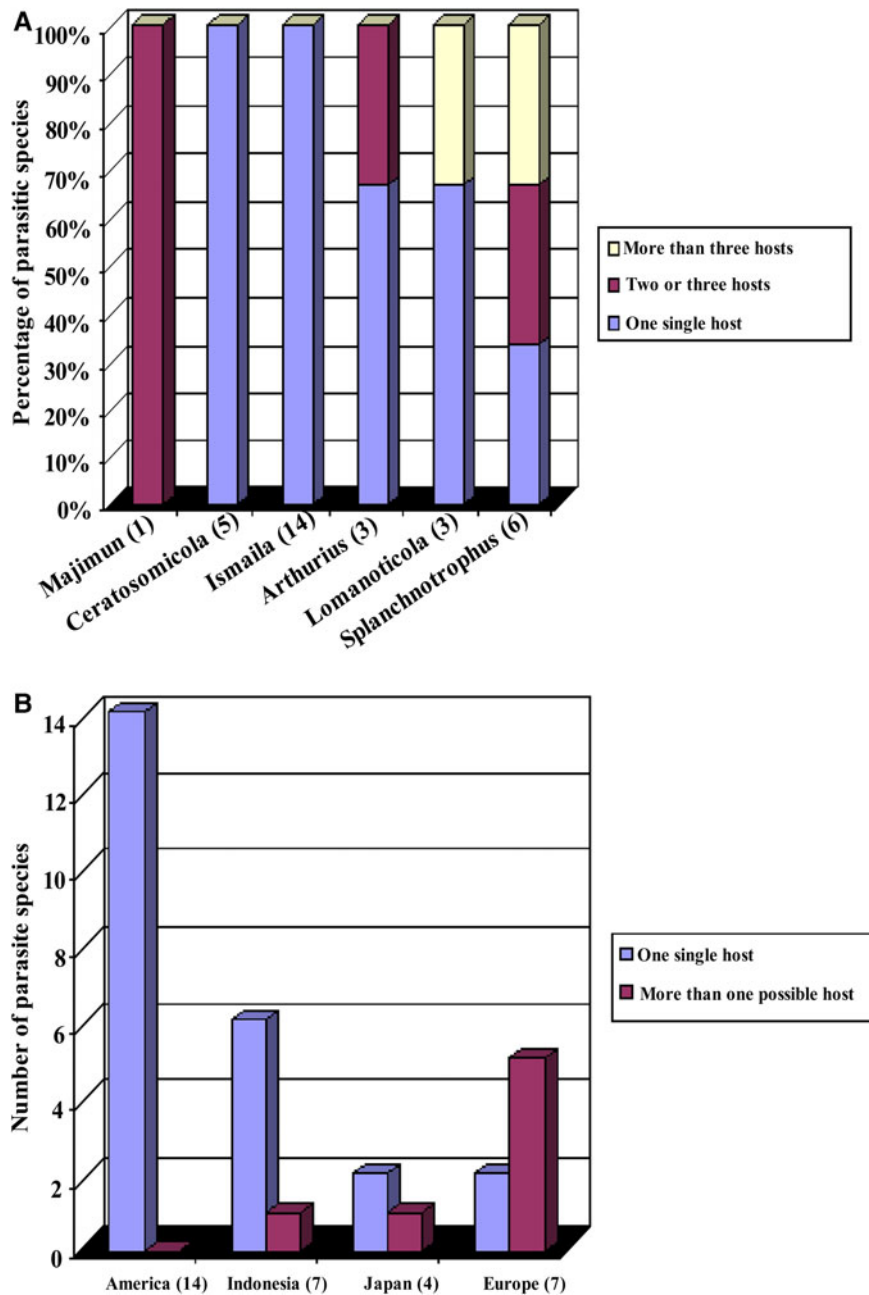
Fig. 1. Overview of the number of host species (A) per splanchnotrophid genus and (B) given the geographic distribution area following Anton & Schrödl (2013a, b). Total number of species given in parentheses.

we applied 94°C – 300 s for the initial step, then 94°C – 45 s, 45°C – 50 s, 72°C – 200 s for 40 cycles, with a final elongation of 72°C – 600 s. For purification of the PCR-product a NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) was used following the manufacturer's instructions. The complete sequencing process was carried out on an ABI 3730 48 capillary sequencer by the Sequencing Service Unit of the Ludwig-Maximilians-University Munich. All sequence amplicons were subjected to a nucleotide BLAST search to test for contamination.

Phylogenetic analysis

COI fragments of 38 splanchnotrophid specimens (12 species from four genera) were obtained. Outgroups included

Pionodesmotes domhainfharraigeanus Anton, Stevenson & Schwabe, 2013 (GenBank accession no. KF652042) and *Cyclopoida* sp. (JX948803.1) (see also Table 1). Consensus sequences were generated with BioEdit (Hall, 1999), edited, translated into amino acid sequences using the invertebrate mitochondrial genetic code, checked for stop codons and frame shifts, and aligned with Muscle using the Mega 5.0 software (Tamura *et al.*, 2011). The alignment then was masked by Gblocks (Castresana, 2000; Talavera & Castresana, 2007) applying less stringent options; substitutional saturation was statistically tested using Dambe (Xia *et al.*, 2003; Xia & Lemey, 2009); base pair frequencies and p-distances were calculated with Mega 5.0.

A maximum likelihood (ML) analysis with 1000 bootstrap (BS) replicates was conducted with RAxML (Stamatakis,

Table 1. Overview of all included specimens giving the registration number, the host specimens and the country and exact location of the collection site respectively.

Voucher ID	GenBank accession number	Species	ZSM-ID	Host	ZSM-ID	Country/Region	Latitude	Longitude	Depth (m)
G 001	KT122805	*Splanchnotrophus angulatus*	ZSMA20142906	*Flabellina ischitana*	ZSM-Mol10100477	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 002	KT122806	*Splanchnotrophus angulatus*	ZSMA20142907	*Spurilla neapolitana*	ZSM-Mol20100409	Croatia/Mala Portic	44°46′45.15″N	13°55′10.84″O	2–5
G 003	KT122807	*Splanchnotrophus angulatus*	ZSMA20142908	*Spurilla neapolitana*	ZSM-Mol20100409	Croatia/Mala Portic	44°46′45.15″N	13°55′10.84″O	2–5
G 004	KT122808	*Splanchnotrophus angulatus*	ZSMA20142909	*Cratena peregrina*	ZSM-Mol20130874	Islote 5.6.1998			
G 005	KT122809	*Splanchnotrophus angulatus*	ZSMA20142910	*Aeolidiella alderi*	ZSM-Mol20070272				
G 006	KT122810	*Ismaila robusta*	inside host	*Phidiana lottini*	ZSM-Mol20110432	Southern Chile/Playa Chica	39°43′10″S	73°24′12″W	2
G 011	KT122812	*Splanchnotrophus angulatus*	ZSMA20142912	*Cratena peregrina*	host lost	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 012	KT122813	*Splanchnotrophus angulatus*	inside host	*Cratena peregrina*	ZSM-Mol20130849	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 013	KT122814	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130850	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 015	KT122815	*Ismaila genalis*	ZSMA20142903	*Holoplocamus papposus*	ZSM-Mol20130872	Southern Chile/Isla Carmen	43°01′08.80″S	72°49′44.79″W	1–20
G 016	KT122816	*Ismaila belciki*	ZSMA20142916	*Janolus fuscus*	host lost	USA/Oregon	43°21′32.4″N	124°18′45.36″W	0–2
G 017	KT122817	*Ismaila volatilis*	ZSMA20142900	*Janolus* spec.	ZSM-Mol20130847	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–20
G 019	KT122818	*Ismaila aliena*	ZSMA20142918	*Thecacera darwini*	ZSM-Mol20130851	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 020	KT122819	*Ismaila aliena*	ZSMA20142919	*Thecacera darwini*	ZSM-Mol20130851	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 021	KT122820	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130852	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 022	KT122821	*Ismaila robusta*	ZSMA20142921	*Phidiana lottini*	host lost	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 023	KT122822	*Ismaila robusta*	ZSMA20142921	*Phidiana lottini*	host lost	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 024	KT122823	*Ismaila robusta*	ZSMA20142923	*Phidiana lottini*	host lost	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 025	KT122824	*Splanchnotrophus angulatus*	inside host	*Spurilla neapolitana*	ZSM-Mol20110684	Italy/Bastione Conca	38°01′03″N	12°30′14″E	2–5
G 028	KT122825	*Ismaila robusta*	ZSMA20142925	*Phidiana lottini*	ZSM-Mol20130855	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 029	KT122826	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130856	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 030	KT122827	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130856	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 031	KT122828	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130857	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 032	KT122829	*Ismaila chaihuiensis*	ZSMA20142902	*Diaulula punctuolata*	ZSM-Mol20130858	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 034	KT122830	*Ismaila damnosa*	ZSMA20142905	*Flabellina* sp. 1	host lost	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	12
G 035	KT122831	*Splanchnotrophus angulatus*	inside host	*Cratena peregrina*	ZSM-Mol20130860	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 036	KT122832	*Splanchnotrophus angulatus*	ZSMA20142930	*Cratena peregrina*	host lost	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 038	KT122833	*Lomanoticola* spec.	ZSMA20142931	*Cuthona cerulea*	ZSM-Mol20130862	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 042	KF652042	*Pionodesmotes domhainfharraigeanus*	ZSMA20130004	*Sperosoma grimaldii*	host lost	Ireland/Whittard Canyon	48.491°N	10.692°W	2000
G 044	KT122834	*Ceratosomicola mammilata*	inside host	*Chromodoris geometrica*	ZSM-Mol20130863	Indonesia/Sulawesi	5°28′29″S	123°45′40″E	4
G 046	KT122835	*Splanchnotrophus gracilis*	ZSMA20142933	*Trapania tartanella*	host lost	Spain/Ria de Ferrol	43°28′02.16″N	8°14′47.70″W	20
G 055	KT122836	*Splanchnotrophus angulatus*	inside host	*Cratena peregrina*	ZSM-Mol20130864	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 056	KT122837	*Splanchnotrophus angulatus*	ZSMA20142935	*Cratena peregrina*	ZSM-Mol20130865	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 057	KT122838	*Ismaila robusta*	ZSMA20142936	*Phidiana lottini*	host lost	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 058	KT122839	*Splanchnotrophus angulatus*	inside host	*Cratena peregrina*	ZSM-Mol20130867	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 059	KT122840	*Ismaila robusta*	ZSMA20142938	*Phidiana lottini*	ZSM-Mol20130868	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 060	KT122841	*Ismaila robusta*	inside host	*Phidiana lottini*	ZSM-Mol20130869	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 082	KT122842	*Ismaila volatilis*	inside host	*Janolus* sp.	ZSM-Mol20130866	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–20
G 100	KT122811	*Ismaila* spec.	inside host	*Eubranchus* sp. 2	ZSM-Mol20130871	Southern Chile/Isla Traiguen	45°11′26.11″S	73°30′49.69″W	6

2014) using the GTRCAT model. Bayesian inference (BI) with MrBayes (Ronquist & Huelsenbeck, 2003) used the invertebrate mitochondrial code, the codon nucleotide model, and 2 million generations, with a sampling frequency of 500 generations. In addition neighbour network graphs were calculated using SplitsTree4 (Huson & Bryant, 2006) to check for incompatibilities within the data.

Detection of barcode gaps, haplotype networks and diagnostic nucleotides

For the genera *Splanchnotrophus* and *Ismaila* a search for barcode gaps was performed using alignments of all sequences of the respective genera and the ABGD-software (Puillandre *et al.*, 2011, 2012), which sorts the sequences into hypothetical species based on the barcode gap, which can be observed whenever the divergence among organisms belonging to the same species is smaller than divergence among organisms from different species. A second approach, Species Identifier (Meier *et al.*, 2006), was used to calculate pairwise distances (see Table 2) and clusters that identify potential species. A third approach was also used, a Poisson Tree Processes (PTP) model (Zhang *et al.*, 2013) provided on the webserver of The Exelixis Lab (URL: http://sco.h-its.org/exelixis/web/software/PTP/index.html), with default settings of 100,000 MCMC generations and a burn-in of 0.1. Furthermore, a statistical parsimony network was conducted on all 13 sequences of *S. angulatus* and on the 19 sequences representing the genus *Ismaila* using the TCS 1.2 software (Clement *et al.*, 2009). Diagnostic characters were obtained through searching the overall alignment following the definition given by Sarkar *et al.* (2008) for single pure and single private characters.

RESULTS

Phylogenetic hypothesis

The final COI alignment consisted of 615 bp, including 38 splanchnotrophid specimens (12 morphologically defined species from four genera) and two outgroup taxa. In Splanchnotrophidae, the mean base pair frequencies for T (34.8%), C (19.5%), A (25.2%) and G (20.5%) reflected the bias towards adenosine and thymine which is characteristic for arthropods (Weis & Melzer, 2012). The index of substitution saturation (Iss) was tested for the whole alignment after Xia & Lemey (2009) with an estimated proportion of invariant sites of 0.54; this was significantly lower than the critical Iss.c value, indicating no substitutional saturation.

Although the neighbour network built with the SplitsTree4 software revealed some conflict within the clades of *Ismaila* and *S. angulatus*, there were very few incompatible splits within the data (Figure 2A). Regarding *Splanchnotrophus*, the specimens parasitizing the nudibranchs *S. neapolitana* and *A. alderi* were recovered as strictly separated to a group including all those utilizing *C. peregrina* or *F. ischitana* as hosts (see Figure 2B). On the other hand, *I. belciki* was recovered as the most basal sister taxon to all other members of the genus. In addition there was split support for a group comprising *I. volatilis*, *Ismaila* sp. and *I. damnosa*, with *I. chaihuiensis* as a basal offshoot (see Figure 2C).

Both ML and BI analyses led to two similar trees, only differing in two regions. In both analyses the Splanchnotrophidae are recovered as a clade with high support (BS 100/BI 1). *Ceratosomicola mammilata* Salmen, Wilson & Schrödl, 2008 formed the highly supported (BS 100/BI 1) sistergroup to the rest, followed by *Splanchnotrophus gracilis* Hancock & Norman, 1863; then all members of *S. angulatus* was recovered as the sister clade to a poorly supported clade formed by *Lomanoticola* and the monophyletic genus *Ismaila* (BS 100/BI 1). Inside the monophyletic (BS 100/BI 1) *S. angulatus* most of the sequences from specimens found in the aeolid nudibranch host *Cratena peregrina* (Facelinidae) clustered together with one sequence from a specimen extracted from the aeolid *Flabellina ischitana* Hirano & Thompson, 1990 (Flabellinidae). However in the ML analysis the clade resulted as a trichotomy consisting of sequence G11, a clade comprising of the three sequences G02, G03 and G25 (infesting the aeolid *Spurilla neapolitana*; Aeolidiidae) together with the sequence G05 (infesting *Aeolidiella alderi* (Cocks, 1852); Aeolidiidae) and a clade with the rest of the sequences as described above (Figure 3). In contrast, the BI analysis recovered a subclade consisting of the sequences G02, G03, G05, G11 and G25 originating from a polytomy formed by the rest of the sequences as described above (Figure 4).

The topologies recovered for the *Ismaila* clade were similar in both analyses with *I. aliena* and *I. robusta* both strongly supported individually and as a sister group. However, the results of the ML analysis suggested a clade with *Ismaila chaihuiensis* Anton, Schories, Jörger, Kalagis & Schrödl, 2015 as its most basal offshoot to a dichotomy of a clade consisting of undescribed *Ismaila* sp. and *Ismaila damnosa* Haumayr & Schrödl, 2003 and a clade comprising *Ismaila volatilis* Anton *et al.*, 2015 and *Ismaila genalis* Anton *et al.*, 2015, forming the sister to the clade of *I. aliena* and *I. robusta* (Figure 3), but with only low support values. In contrast, BI favoured a polytomy of *I. volatilis*, *I. genalis*, a clade comprising *I. damnosa* and *Ismaila* sp. and a dichotomy of *I. aliena* and *I. robusta*. Within *I. robusta* three sequences (G22, G24 & G28) formed a subclade with moderate support (BS62/BI96, see also Figure 4) in both analyses.

Distances and barcode gaps

P-distances between the included splanchnotrophid genera are given in Table 2. Within genera the ABGD-analyses revealed strong barcode gaps. In *Ismaila*, ABGD favoured five groups: group 1 consists of *Ismaila* sp., *I. genalis*, *I. volatilis* and *I. damnosa*; group 2 represents *I. robusta*; group 3 represents *I. aliena*; group 4 *I. belciki* and group 5 *I. chaihuiensis*. For the genus *Splanchnotrophus* the ABGD-analyses also revealed a strong barcode gap between *S. angulatus* and *S. gracilis*, but between $P = 0.0010$ and $P = 0.0046$ the sequences formed three different groups, with two sequences separated from the rest of the *S. angulatus* group. Excluding *S. gracilis*, ABGD still favoured this split within *S. angulatus*; however, there is no clear detectable barcode gap.

The software Species Identifier found 12 clusters, under a threshold of 2.42%, calculated from a pairwise summary. Clusters 1 and 10 represent the two outgroup taxa. Cluster 2 included all *S. angulatus* sequences and cluster 3 represented *I. robusta*. Cluster 4 included all sequences of *I. volatilis*, *Ismaila* sp. and *I. damnosa*. Clusters 5, 6, 7, 8 and 9 represented the species *I. aliena*, *I. genalis*, *I. belciki*, *I. chaihuiensis*

Table 2. Data output of pairwise distances calculated with SPECIES IDENTIFIER.

| Sequence name	Largest conspecific match	Distance	Overlap	Closest congeneric. interspecific match	Distance	Overlap
Cyclopoida sp.	No matching conspecific sequence	N/A	N/A	No matching congeneric, interspecific sequence	N/A	N/A
G01 *Splanchnotrophus angulatus* F	G03 *Splanchnotrophus angulatus* S	2.19	638	G46 *Splanchnotrophus gracilis*	15.55	643
G02 *Splanchnotrophus angulatus* S	G12 *Splanchnotrophus angulatus* C	2.73	657	G46 *Splanchnotrophus gracilis*	16.19	667
G03 *Splanchnotrophus angulatus* S	G12 *Splanchnotrophus angulatus* C	2.73	657	G46 *Splanchnotrophus gracilis*	16.46	662
G04 *Splanchnotrophus angulatus* C	G03 *Splanchnotrophus angulatus* S	2.42	660	G46 *Splanchnotrophus gracilis*	15.6	660
G05 *Splanchnotrophus angulatus* A	G03 *Splanchnotrophus angulatus* S	1.81	662	G46 *Splanchnotrophus gracilis*	14.39	667
G06 *Ismaila robusta*	G57 *Ismaila robusta*	0.62	639	G13 *Ismaila aliena*	4.06	639
G100 *Ismaila* sp.	No matching conspecific sequence	N/A	N/A	G34 *Ismaila damnosa*	1.48	672
G11 *Splanchnotrophus angulatus* C	G03 *Splanchnotrophus angulatus* S	2.11	662	G46 *Splanchnotrophus gracilis*	15.14	667
G12 *Splanchnotrophus angulatus* C	G03 *Splanchnotrophus angulatus* S	2.73	657	G46 *Splanchnotrophus gracilis*	15.67	657
G13 *Ismaila aliena*	G21 *Ismaila aliena*	0.44	671	G23 *Ismaila robusta*	4.01	672
G15 *Ismaila genalis*	No matching conspecific sequence	N/A	N/A	G06 *Ismaila robusta*	5.63	639
G16 *Ismaila belciki*	No matching conspecific sequence	N/A	N/A	G100 *Ismaila* sp.	12.2	672
G17 *Ismaila volatilis*	G82 *Ismaila volatilis*	2.1	666	G34 *Ismaila damnosa*	1.63	671
G19 *Ismaila aliena*	G21 *Ismaila aliena*	0.74	669	G23 *Ismaila robusta*	4.33	669
G20 *Ismaila aliena*	G21 *Ismaila aliena*	0.59	671	G23 *Ismaila robusta*	4.17	671
G21 *Ismaila aliena*	G19 *Ismaila aliena*	0.74	669	G59 *Ismaila robusta*	4.39	592
G22 *Ismaila robusta*	G57 *Ismaila robusta*	0.59	672	G13 *Ismaila aliena*	4.16	672
G23 *Ismaila robusta*	G57 *Ismaila robusta*	0.44	672	G13 *Ismaila aliena*	4.01	672
G24 *Ismaila robusta*	G57 *Ismaila robusta*	0.89	671	G13 *Ismaila aliena*	4.17	671
G25 *Splanchnotrophus angulatus* S	G03 *Splanchnotrophus angulatus* S	2.41	662	G46 *Splanchnotrophus gracilis*	15.59	667
G28 *Ismaila robusta*	G57 *Ismaila robusta*	0.74	672	G13 *Ismaila aliena*	4.31	672
G29 *Ismaila aliena*	G21 *Ismaila aliena*	0.44	671	G23 *Ismaila robusta*	4.01	672
G30 *Ismaila aliena*	G21 *Ismaila aliena*	0.44	671	G23 *Ismaila robusta*	4.01	672
G31 *Ismaila aliena*	G21 *Ismaila aliena*	0.74	670	G06 *Ismaila robusta*	4.22	639
G32 *Ismaila chaihuiensis*	No matching conspecific sequence	N/A	N/A	G82 *Ismaila volatilis*	3.74	667
G34 *Ismaila damnosa*	No matching conspecific sequence	N/A	N/A	G100 *Ismaila* sp.	1.48	672
G35 *Splanchnotrophus angulatus* C	G03 Splanchnotrophus *angulatus* S	1.96	662	G46 *Splanchnotrophus gracilis*	15.14	667
G36 *Splanchnotrophus angulatus* C	G03 Splanchnotrophus *angulatus* S	2.11	662	G46 *Splanchnotrophus gracilis*	14.99	667
G38 *Lomanoticola* sp.	No matching conspecific sequence	N/A	N/A	No matching congeneric, interspecific sequence	N/A	N/A
G42 *Pionodesmotes domhainfharraigeanus*	No matching conspecific sequence	N/A	N/A	No matching congeneric, interspecific sequence	N/A	N/A
G44 *Ceratosomicola mammillata*	No matching conspecific sequence	N/A	N/A	No matching congeneric, interspecific sequence	N/A	N/A
G46 *Splanchnotrophus gracilis*	No matching conspecific sequence	N/A	N/A	G05 *Splanchnotrophus angulatus* A	14.39	667
G55 *Splanchnotrophus angulatus* C	G03 *Splanchnotrophus angulatus* S	2.26	661	G46 *Splanchnotrophus gracilis*	14.86	666
G56 *Splanchnotrophus angulatus* C	G03 *Splanchnotrophus angulatus* S	1.96	662	G46 *Splanchnotrophus gracilis*	15.14	667
G57 *Ismaila robusta*	G24 *Ismaila robusta*	0.89	671	G13 *Ismaila aliena*	4.46	672
G58 *Splanchnotrophus angulatus* C	G03 *Splanchnotrophus angulatus* S	2.43	658	G46 *Splanchnotrophus gracilis*	15.23	663
G59 *Ismaila robusta*	G24 *Ismaila robusta*	0.5	592	G13 *Ismaila aliena*	4.22	592
G60 *Ismaila robusta*	G57 *Ismaila robusta*	0.44	672	G13 *Ismaila aliena*	4.01	672
G82 *Ismaila volatilis*	G17 *Ismaila volatilis*	2.1	666	G34 *Ismaila damnosa*	2.24	667
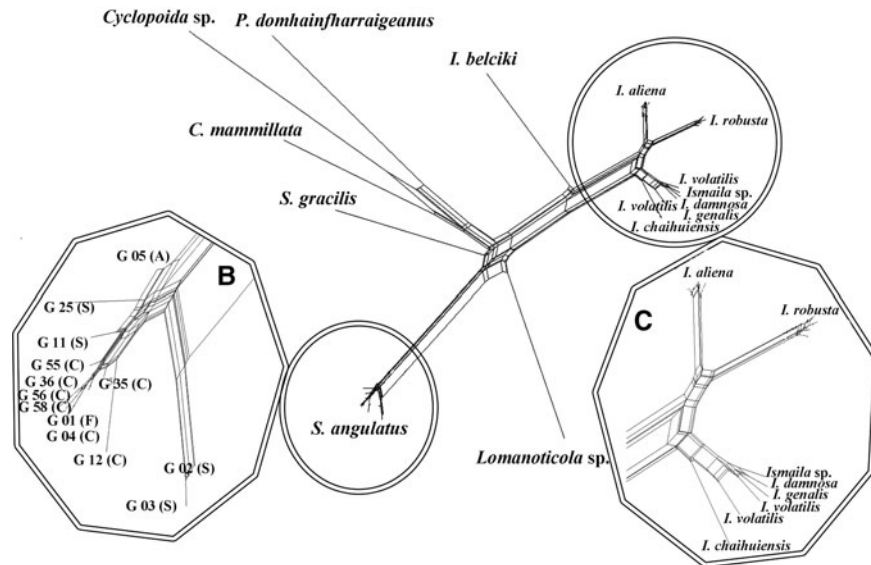
Fig. 2. Neighbour network computed by SplitsTree (A) with magnifications for the regions of interest inside (B) the *Ismaila* and (C) the *Splanchnotrophus* cluster. Capitals before or following species name refer to respective hosts: C: *Cratena peregrina*; S: *Spurilla neapolitana*; F: *Flabellina ischitana*; A: *Aeolidiella alderi*.

and *Lomanoticola* sp. respectively. Cluster 11 included *C. mammillata* and cluster 12 *S. gracilis* (see Figure 5G).

The PTP-analysis indicated outgroup taxa, *C. mammillata, S. gracilis, Lomanoticola* sp. and *I. belciki* as independent

species with high support values. Good support was recognized for *S. angulatus, I. aliena* and *I. robusta*. However, all recently discovered *Ismaila* species form one cluster, although this is poorly supported (Figure 5F). Results are mostly
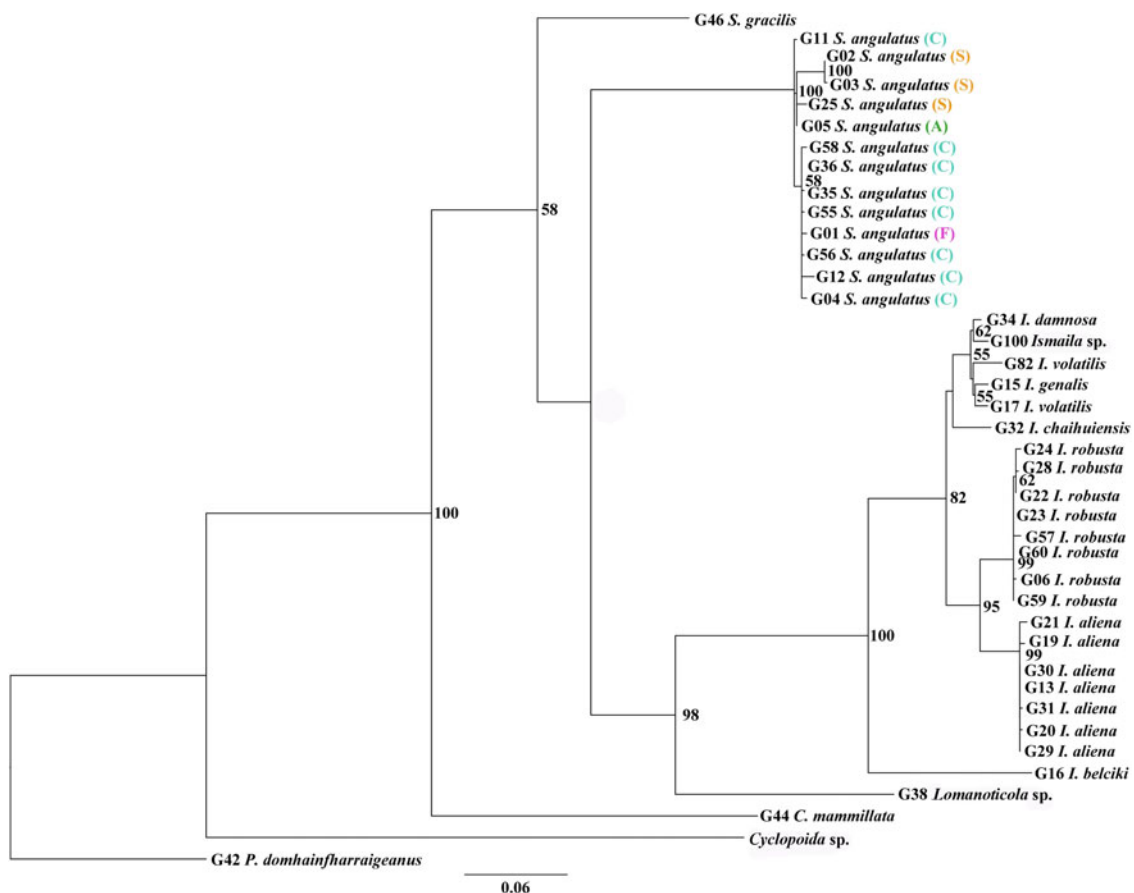
Fig. 3. Maximum likelihood consensus tree of the cytochrome c oxidase I (COI) sequences of 38 splanchnotrophids and two outgroup taxa. Numbers above branches show bootstrap values (>55%); branch length indicates substitutions per site. Capitals in parentheses refer to respective hosts: C: *Cratena peregrina*; S: *Spurilla neapolitana*; F: *Flabellina ischitana*; A: *Aeolidiella alderi*.
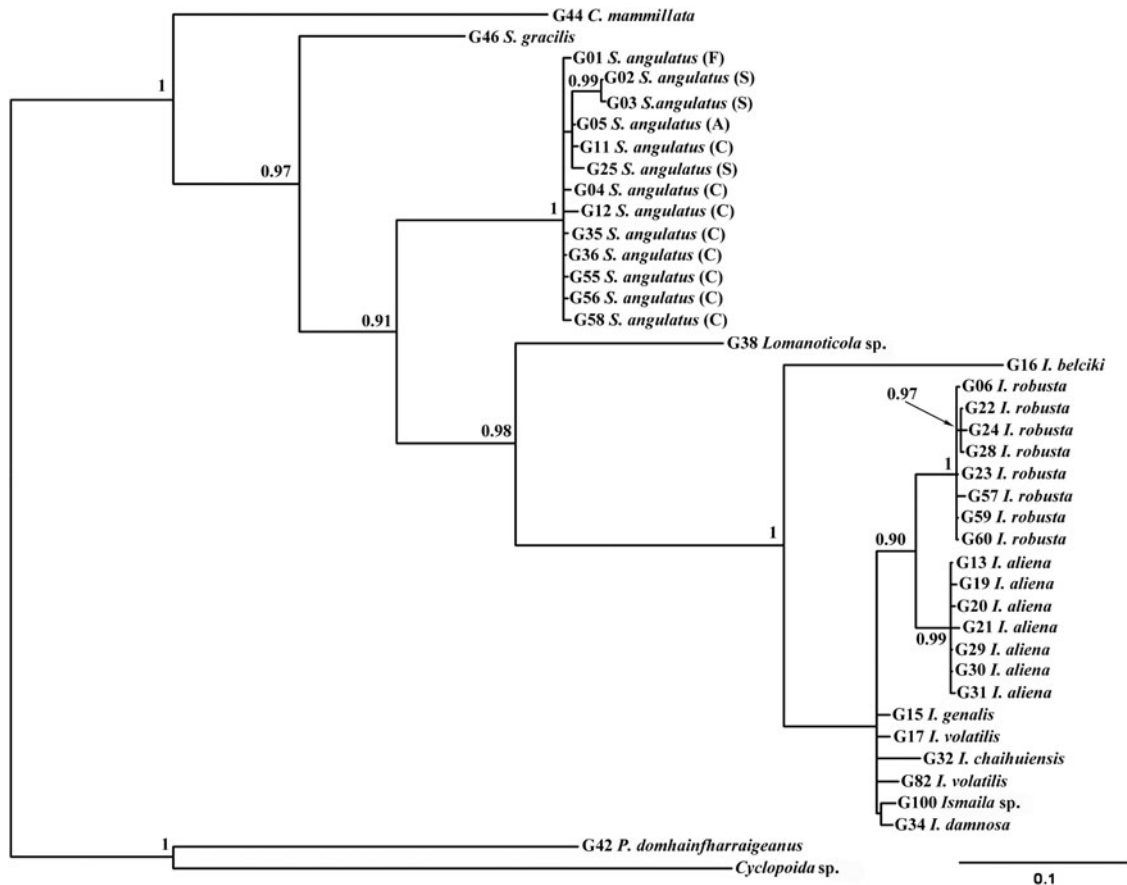
Fig. 4. Bayesian inference consensus tree of the cytochrome c oxidase I (COI) sequences of 38 splanchnotrophids and two outgroup taxa. Numbers above branches show posterior probability of BI (>0.90); branch length indicates substitutions per site. Capitals in parentheses refer to respective hosts: C: *Cratena peregrina*; S: *Spurilla neapolitana*; F: *Flabellina ischitana*; A: *Aeolidiella alderi*.

congruent regarding the ML and BI approach implemented in the PTP-analysis. Differences include the clade containing *Ismaila* sp. emerging as one species in the ML approach, while *I. genalis*, *I. chaihuiensis* and one sequence of *I. volatilis* (G17) are recovered as distinct species in the BI approach (see Figure 5F).

Haplotype networks

Each of the 13 *S. angulatus* sequences represented a distinct haplotype. The analysis using TCS software with a 90% statistical parsimony connection limit led to one network linking all haplotypes. In this network the inferred ancestral haplotype was from the host *Cratena peregrina*. Other haplotypes from this host were connected nearby (except G4 and G12), whereas those infesting other host species occupied more derived positions (Figure 6). However, setting the statistical parsimony connection limit to 95%, as is usually applied, resulted in three separate networks (see Figure 7). The first consisted of two sequences from the host *Cratena peregrina* and the second consisted of the two haplotypes G02 and G03 (infesting *Spurilla neapolitana*). The third network comprised the rest of sequences, with all sequences from haplotypes infesting *Cratena peregrina* inferred to be more ancestral and the haplotypes of three specimens infesting other hosts occurred in the more derived positions (Figure 7).

For the genus *Ismaila*, i.e. *I. belciki, I. aliena, I. genalis* and *I. chaihuiensis* were recovered as independent networks under a 95% statistical parsimony connection limit. Although most haplotypes of *I. robusta* emerged as a single network, there were two haplotypes (G06 and G59) that separated into an independent haplotype network. Another independent network consisted of a single haplotype shared by *I. volatilis, Ismaila* sp. and *I. damnosa*. However the second included haplotype of *I. volatilis* formed a separate network (Figure 8).

Diagnostic nucleotides

Splanchnotrophus gracilis differed from *S. angulatus* in 81 single pure characters (following Sarkar *et al.*, 2008; Jörger & Schrödl, 2014). *Lomanoticola* sp. differed from the genus *Splanchnotrophus* in 40 single pure characters. Within the genus *Ismaila, I. belciki* showed the highest divergence with 31 single pure characters differing from other *Ismaila* species. *Ismaila robusta* differed in nine, *I. aliena* in six, *I. chaihuiensis* in five, *I. genalis* in four and *I. damnosa, Ismaila* sp. and *I. volatilis* in one single pure character respectively.

Inside *S. angulatus* there were no differing single pure characters discernable; however, those parasites extracted from the host *C. peregrina* differed in nine single private characters from those infesting other host species. In addition, the *S.*
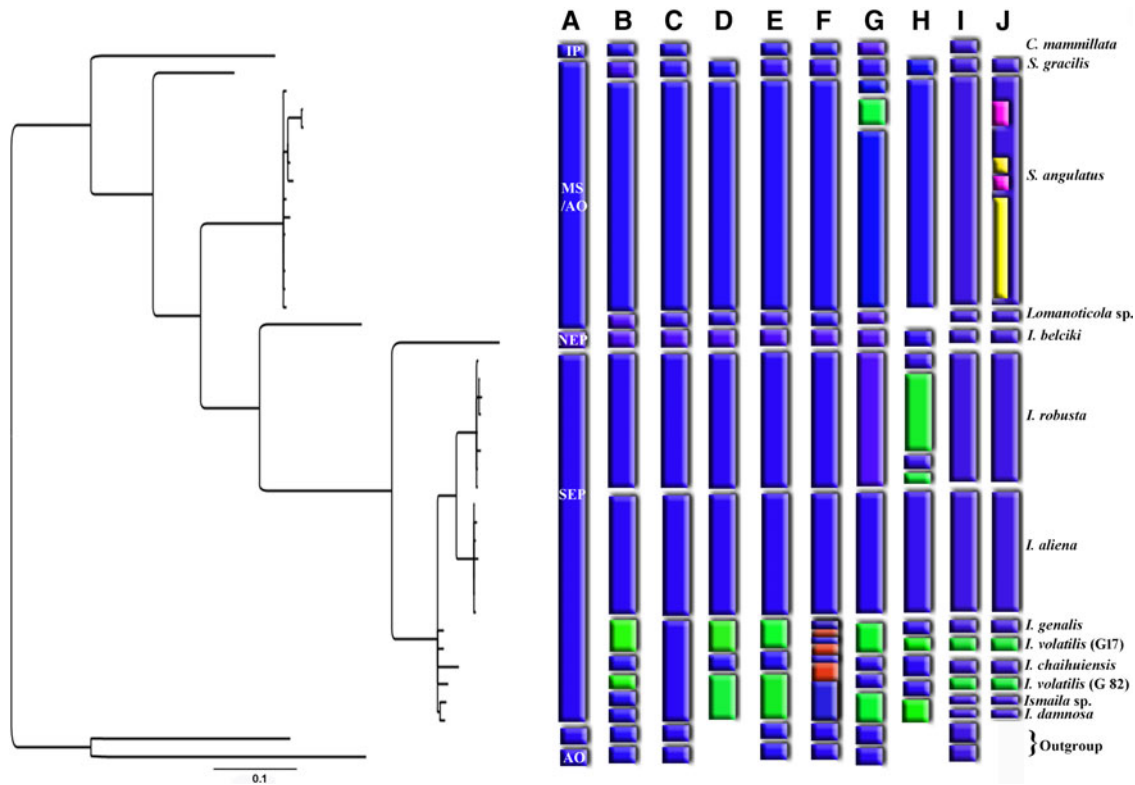
Fig. 5. Geographic distribution, sequence clusters and potential species obtained with the respective methods plotted in the Bayesian Inference tree. (A) Geographic distribution: IP, Indo-Pacific; MS, Mediterranean Sea; AO, Atlantic Ocean; NEP, north-eastern Pacific; SEP, south-eastern Pacific; (B) Maximum likelihood; (C) Bayesian inference; (D) ABGD; (E) Species Identifier; (F) PhyloMap – Poisson Tree Processes (PTP). The blue bars represent congruent results of the ML/BI approach, while the red bar indicates the differing results of the ML/BI approach; (G) Splitstree; (H) Tcs independent parsimony haplotype networks; (I) traditional species hypotheses based on morphological characters; (K) diagnostic nucleotides. Bars represent clades. Green bars represent clades in the respective analysis, which are not represented in the Bayesian Inference tree. Yellow and pink bars indicate groups within *S. angulatus* infesting *S. neapolitana* (pink) and *C. peregrina* (yellow) differing only in single private characters.

angulatus found in *S. neapolitana* also differed in nine single private characters from all other conspecifics. These nine single private characters did not overlap.

DISCUSSION

The high species diversity of copepods makes morphological identification and quantification of species a challenging task (Blanco-Berical et al., 2014). In such cases DNA barcoding can be a simple but suitable tool to help identify species and to shed at least some light at the respective relationships (Blanco-Berical et al., 2014; Jörger et al., 2014; Padula et al., 2014). However, barcoding identification requires that the taxonomy of the group is known, and that these taxonomic units correspond to a clade of COI sequences. This is the first attempt to apply molecular techniques to members of the Splanchnotrophidae to test the current morphology-based species hypotheses and to study the host specificity of selected members of the family.

Phylogeny of the Splanchnotrophidae

The resulting molecular trees are generally congruent with the current morphocladistic hypotheses on splanchnotrophid phylogeny (Anton & Schrödl, 2013a, b). The traditionally accepted monophyly of Splanchnotrophidae (e.g. Huys, 2001) is supported here, as is the monophyly of the

Panamerican genus *Ismaila*. *Splanchnotrophus*, another morphology-based genus represented herein with multiple individuals, appeared paraphyletic. Surprisingly, *S. gracilis*, infesting the dorid nudibranch *Trapania tartanella* (Ihering, 1886), was recovered sister to all splanchnotrophids but *Ceratosomicola*. The COI topologies (Figures 3 & 4) suggested *Ceratosomicola* as earliest splanchnotrophid offshoot, which is also in accord with the results of the morphocladistic analyses of Anton & Schrödl (2013a, b). Interestingly, *Ismaila* is sister to *Lomanoticola* in the molecular trees, while morphological data usually suggested a clade of *Splanchnotrophus* and *Lomanoticola*. This supports Huys (2001) who elevated *Lomanoticola*, which was previously considered a subgenus of *Splanchnotrophus* (Hecht, 1895; Monod & Dollfus, 1932; Delamare Debouteville, 1950; Jensen, 1990), to genus rank. Obviously, future molecular analyses should include further splanchnotrophid species, covering the entire generic, morphological and geographic diversity of the family, and representatives of *Briarella*, the putative sister of Splanchnotrophidae. As indicated by high support values, the barcoding fragment of COI appears informative for resolving splanchnotrophid genus level phylogeny.

On a species level, molecular phylogenetic trees are compatible with traditional taxonomy, but do not resolve all of the valid parasite species based on morphology. COI trees confirm the monophyly of *S. angulatus* and its separation from *S. gracilis* (Figures 2 – 5) as already suggested by previous studies based on morphological data (Huys, 2001; Abad et al.,
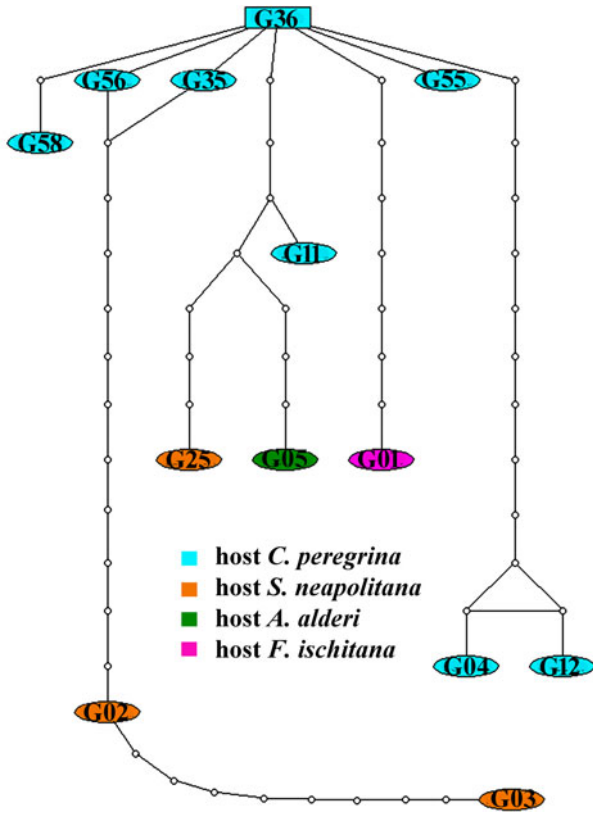
Fig. 6. Statistical parsimony network of 13 COI haplotypes in *Splanchnotrophus angulatus* with a connection limit set to 90%; white dots represent intermediate haplotypes missing in the sample set.

2011; Anton & Schrödl, 2013a, b). Within *Ismaila*, the morphologically clearly distinct species *I. robusta*, *I. aliena* and *I. belciki* were recovered monophyletic, while the recently described and similarly characteristic *I. volatilis* was not. The remaining species *I. genalis*, *I. chaihuiensis*, *Ismaila* sp. and *I. damnosa* emerged as a common clade in the ML analysis but paraphyletic in the BI analysis.

Phylogenetic trees showing a characteristic branching pattern with long internodes leading to well-supported shallow nodes with a couple of short terminals are often
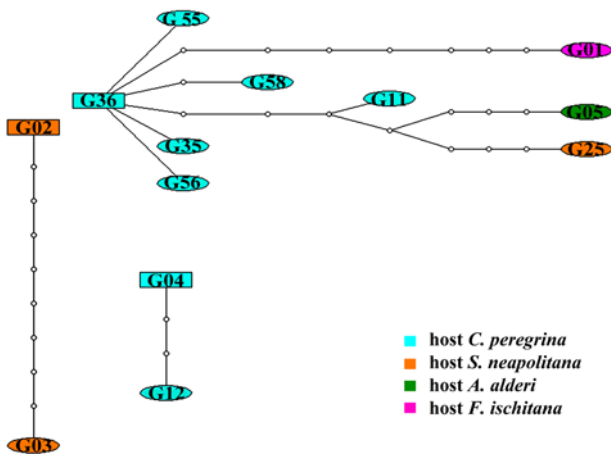

Fig. 7. Statistical parsimony network of 13 COI haplotypes in *Splanchnotrophus angulatus* with a connection limit of 95%; white dots represent intermediate haplotypes missing in the sample set.
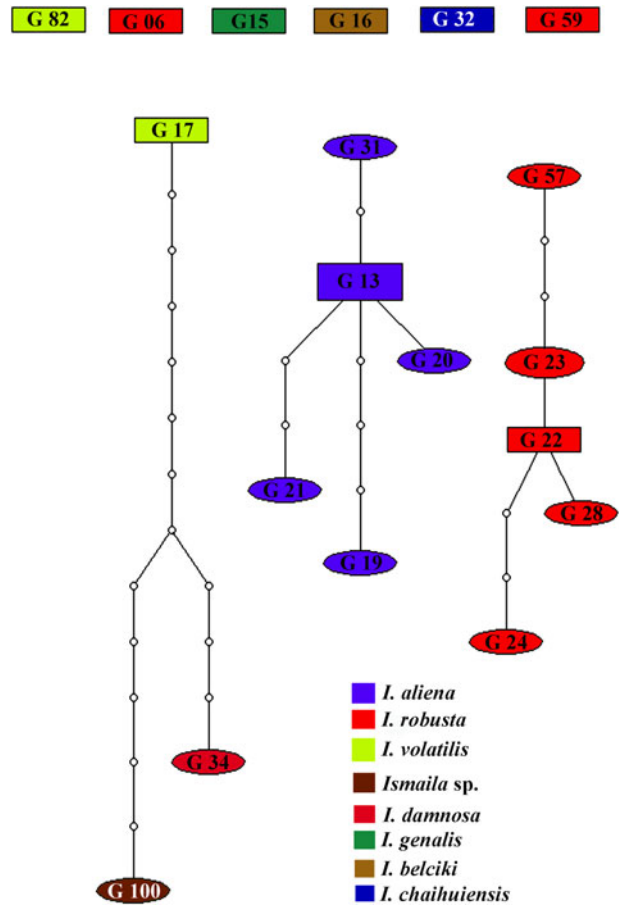

Fig. 8. Statistical parsimony network of 19 haplotypes of the genus *Ismaila* with a connection limit of 95%; white dots represent intermediate haplotypes missing in the sample set.

believed to be suggestive for species units, although there is no objective way to interpret the meaning of such units and their potential substructure appropriately by eye. In current barcoding practice, even a distance-based, quickly calculated COI genealogy, combined with some genetic threshold value, may deliver a first approximation on potential species (e.g. Layton *et al.*, 2014), and this may be useful to get a rough estimate on species diversity, e.g. when dealing with rare(ly sampled) groups or remote habitats (Jörger *et al.*, 2010, 2014; Padula *et al.*, 2014). However, gene histories may differ, and splanchnotrophid species level relationships appear to be complicated. Our initial phylogenetic, species delimitation and network analyses herein are based on a single gene and on an incomplete taxon and population sampling, and are inevitably preliminary.

Molecular species delimitation

Regarding *Splanchnotrophus*, both Species Identifier and ABGD basically confirmed the two morphological species *S. gracilis* and *S. angulatus* (Figure 5), showing considerable minimum interspecific p-distance of 16.4%. This is also supported by the presence of 81 single pure diagnostic characters and the results of the PTP-analysis (Figure 5F). However, two of the three *Spurilla* infesting *S. angulatus* animals isolated from the same host individual were separated under certain ABGD permutations. The hypothesis of a third,

morphologically cryptic *Splanchnotrophus* species is supported by the haplotype network analysis (Figures 6–8), since recovering separate networks using a 95% connection limit is sometimes used as a predictor of speciation; e.g. Miralles *et al.* (2011) considered species as distinct if showing separate mtDNA haplotype networks and unshared nDNA haplotypes. Unfortunately there are no reliable data from nuclear markers available for the Splanchnotrophidae.

According to the presence and number of diagnostic nucleotides both *S. gracilis* and *Lomanoticola* sp. receive good support. Regarding *Ismaila*, *I. belciki* is clearly separated from *I. robusta* and *I. aliena* also supported by differences in 31 single pure diagnostic characters. Within the genus, however, there is only poor support for the included species regarding diagnostic nucleotides. With a maximum of nine single pure characters *I. robusta* gains the highest support, but *I. damnosa*, *Ismaila* sp. and *I. volatilis* differ only in one single pure character respectively. Regarding *S. angulatus* there are no differences in single pure characters detectable according to the respective host species, supporting the hypothesis of one species displaying a lower level of host specificity. However the nine independent single private characters found for those individuals infesting *S. neapolitana* and those infesting *C. peregrina* respectively seem to indicate some kind of autocorrelation between gene flow and host.

In contrast to the ambiguous phylogenetic analyses, ABGD indicates *I. chaihuiensis* as a distinct species also (Figure 5D).

Ismaila aliena, *I. chaihuiensis*, *I. belciki* and *I. genalis* are supported as distinct species by the results of the TCS analysis (Figures 5H–8), since they all were recovered as independent networks or independent haplotypes, respectively. *Ismaila robusta* is also supported, nevertheless two sequences emerged as independent haplotypes (Figure 5H). In the case of G06 a possible explanation for this separation could be the geographic origin of the sample, which is quite distant to the location of all the other samples of *I. robusta* (see Table 1). G59, however, was collected in the same location as the rest of the specimens, so the separation from the other haplotypes remains unexplained. Neither changing the connection limit nor excluding any other haplotype had any influence on the result. The large number of inferred extinct or unsampled haplotypes suggests the data set is highly undersampled, which can result in inferring more structure than is actually present.

Ismaila damnosa, *Ismaila* sp. and *I. volatilis* emerging in the same haplotype network might initially seem to contradict the hypothesis of independent species. However, these three species are each represented only by a single sequence, rendering any attempt of estimating the intra- or interspecific variation impossible. Only a single pure diagnostic character supports these three species respectively, but this may also change as data increase. At the present time, at least some diagnostic nucleotides were found for all included *Ismaila* species; future exploration of the quantity and significance of diagnostic characters needs more genetic material, and the validity of these species remains somewhat equivocal.

Host specificity: *Ismaila* versus *Splanchnotrophus*

Of the morphology-defined *Ismaila* species included in the molecular analyses, the specific status of *I. aliena* and *I.* *robusta* was unambiguously confirmed. Both *Ismaila aliena* and *I. robusta* were previously assumed to be strictly host specific (to the dorid nudibranchs *Thecacera darwini* Pruvot-Fol, 1950 and *Okenia luna* Millen, Schrödl, Vargas & Indacochea, 1994, repectively), and this is supported herein. Assessing the specificity of the remaining *Ismaila* species is much harder since there are so few observations. The limited barcoding data to date remains compatible with assuming strict host specificity of the herein included *I. belciki, I. damnosa, I. genalis, I. volatilis, I. chaihuiensis* and *Ismaila* sp. (Figure 5). This null hypothesis of specificity was generated by the state being plesiomorphic in the phylogenetic hypothesis of Anton & Schrödl (2013a), and in light of our initial molecular data, there is no reason yet to assume host-induced morphological plasticity in *Ismaila*. We conclude that the earlier hypothesis of a species-rich neotropical clade *Ismaila* showing a rather rapid and recent radiation via host switches (Schrödl, 2003; Anton & Schrödl, 2013a, b) remains a plausible evolutionary scenario.

Splanchnotrophus angulatus was recovered as a single species in both phylogenetic analyses (Figures 3 & 4). There is no genetic substructure suggestive of a hidden species complex according to the ABGD analysis, which showed no distinct barcode gap for *S. angulatus*. In the light of barcoding data, *S. angulatus* is a single species infesting various host species, including the aeolids *Spurilla neapolitana, Aeolidia alderi, Cratena peregrina* and *Flabellina ischitana*, comprising three different host families. Interestingly, two of three members of *S. angulatus* infesting *Spurilla neapolitana* cluster together in both phylogenetic analyses. This subgroup is also supported by the results of the ABDG- and TCS analyses (Figures 6–8). This genetically derived group may reflect some reproductive isolation due to distinct host species and represents a beginning state of speciation. According to the results of the TCS analysis there is also another group separating from the rest, consisting of two haplotypes infesting *Cratena peregrina*. Nevertheless, divergences are low, ABGD analyses show no distinct barcode gap, and only single private characters were found, suggestive of early divergence or limited gene flow due to ecological host differences. Morphological comparisons thus are overdue to scrutinize current taxonomy, and they need to be on a broader basis, i.e. revising all relevant *Splanchnotrophus* type material and specimens from a broad range of hosts.

The different life-history strategies and their potential reasons

All members of the Splanchnotrophidae capable of infesting more than two host species were reported from the Mediterranean Sea and the European coasts of the Atlantic Ocean (Figure 1), and all belong to the genus *Splanchnotrophus* in a broad sense. Huys (2001) split *Lomanoticola* from *Splanchnotrophus*, and both were considered either sister taxa or *Splanchnotrophus* deriving from paraphyletic *Lomanoticola* (Anton & Schrödl, 2013a). Regardless, the ability to infest several, not necessarily closely related hosts, appeared phylogenetically and geographically correlated. Morphocladistic and molecular tree hypotheses all support a scenario in which ancestral splanchnotrophid lineages, *Ceratosomicola, Ismaila* and *Arthurius* are highly specific to a single host. Assuming diversification via

host switch in *Ismaila* (Anton & Schrödl, 2013a, b), infestation of a new host seems to invariably reduce or lose the ability to infest the original host, thus creating a bottleneck leading to a reproductive barrier. An obvious consequence of this scenario, if confirmed, is that strictly host-specific lineages can radiate in sympatry, adapting to different hosts. Strict dependence on certain sea slug hosts, which may be highly sporadic or rare (Schrödl, 2003), means higher risk of rapid extinction of newly diversified parasites. In contrast, host-promiscuous *Lomanoticola* and *Splanchnotrophus*, if confirmed by morphology-based taxonomy, may need allopatry to diverge permanently, and would have a lowered extinction risk.

CONCLUSION

The present study successfully extracted genetic material from the egg sacs of female parasites, with minimal damage of rare specimens (Anton *et al.*, 2013). Our preliminary molecular study on splanchnotrophids included 11 of the currently 32 known species and a new *Ismaila* sp., many with single or few specimens; the need for more samples and markers thus is obvious. These first molecular-based analyses are largely but not fully congruent with morphology-based taxonomic hypotheses on Splanchnotrophidae (Figure 5). In addition, host specificity reported *Splanchnotrophus*, could be confirmed. Amphi-American *Ismaila* appears to radiate via host switches, losing connection to ancient populations, while individuals of *Splanchnotrophus angulatus* infesting different hosts may maintain some gene exchange. Uncovering details, reasons and consequences of these substantially different ecological and evolutionary strategies in the family Splanchnotrophidae provides an interesting field of research. In addition to morphology-based taxonomic revisions, we need more information on the life cycles of splanchnotrophids, on mechanisms of infections and on population dynamics of parasites and hosts to understand coevolution.

CONFLICT OF INTEREST

None.

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