Supplement 1

Text S1: Additional supporting molecular evidence from the ITS2 rDNA region

The ITS rDNA region is highly effective for resolving digenean species as it has greater variation within the generic taxonomic level in this group than other barcoding genes (Nolan & Cribb 2005). Since very little to no genetic variation was observed in the 18S and 28S regions among the newly discovered vent digenean morphogroups in our study, we sequenced ITS2 to further explore whether they might indeed represent distinct species or should be lumped into a single species.

The same extraction, sequencing, sequence alignment, and tree generation methods were used as in the main manuscript, with the following PCR modifications for the ITS2 region: ITS2 rDNA was amplified with the primers 3S (5'-GGT ACC GGT GGA TCA CGT GGC TAG TG-3') (Morgan & Blair 1995) and ITS2.2 (5'-CCT GGT TAG TTT CTT TTC CTC CGC-3') (Cribb et al. 1998), following the protocol from Martin et al. (2017): 1x (3 min at 95°C, 2 min at 45°C, 90 sec at 72°C), 4x (45 sec at 95°C, 45 sec at 50°C, 90 sec at 72°C), 30x (20 sec at 95°C, 20 sec at 52°C, 90 sec at 72°C), 1x (5 min extension only at 72°C). As with 18S and 28S, successful amplification was confirmed with gel electrophoresis and PCR product was sent to Sequegen DNA Sequencing (Worcester, Massachusetts) for Sanger sequencing in both the forward and reverse directions. Chromatograms were used to clean sequences, and consensus sequences were created using Sequencher Ver. 5.4.6 (Gene Codes Corporation, Ann Arbor, Michigan).

Results

ITS2 confirmed that all individuals in the *Neolebouria* morphogroup DIGE08 are genetically identical and are identical to the sporocyst SPOR01 (Fig. S1a). Morphogroups in the genera *Caudotestis* and *Biospeedotrema* showed 0.2–1.0% genetic variation between morphogroups (Table S1, Fig. S1b). The morphogroups DIGE01, DIGE13, and DIGE20 did not show evidence of intraspecific variation, but DIGE10 and META01 had two separate genetic clusters, which could have arisen from erroneous morphological sorting. These morphogroups did not cluster with other sequenced morphogroups, indicating there may be additional vent digenean diversity not captured by our morphological sorting. Some META01 individuals were genetically identical to DIGE13, consistent with 18S and 28S analyses. The metacercaria META08 was 0.2% dissimilar from DIGE01, indicating these might not be an exact match but are in the same genus. The number of sequences obtained per morphogroup and their GenBank accession numbers are presented as a spreadsheet in the electronic supplementary materials.

Table S1. Percent identity and number of base pairs of aligned genetic data, after removal of all gaps, comparing the ITS2 sequence of DIGE 13 to the other morphogroups in *Caudotestis* and *Biospeedotrema*. Note that the morphogroups DIGE10 and META01 were split, so the two groups are labeled as "cluster a" and "cluster b" corresponding to the tree in Figure S1b.

Morphogroup	**Percent Identity**	**Percent Difference**	**Base Pairs**
Biospeedotrema DIGE13	100	0	435/435
Biospeedotrema META01 – cluster a	100	0	435/435
Biospeedotrema DIGE10 – cluster b	99.8	0.2	434/435
Biospeedotrema DIGE20	99.8	0.2	434/435
Caudotestis META08	99.3	0.7	432/435
Biospeedotrema DIGE10 – cluster a	99.0	1.0	431/435
Caudotestis META01 – cluster b	99.0	1.0	431/435
Caudotestis DIGE01	99.0	1.0	431/435

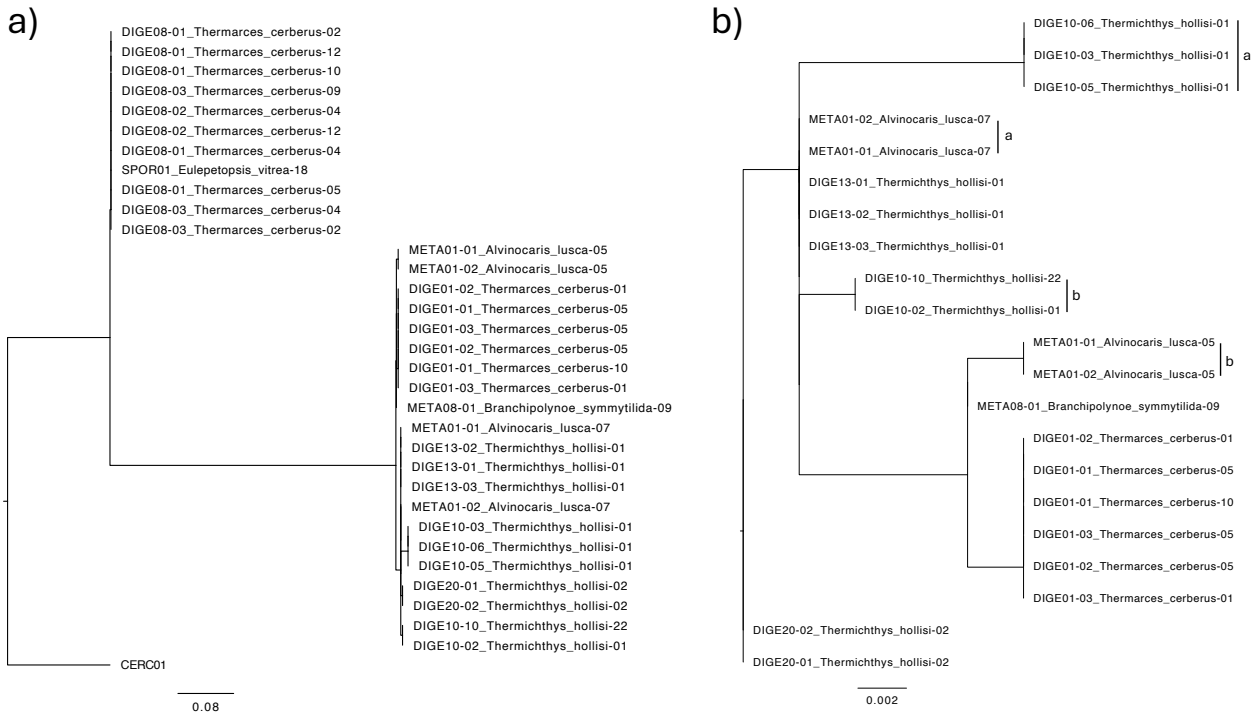

Figure S1. (a) Phylogenetic trees resulting from maximum likelihood analysis of the 392 bp partial ITS2 rDNA alignment including individuals of all successfully sequenced vent digenean morphogroups and life stages within the genera *Neolebouria* (Morphogroups: DIGE08, SPOR01), *Biospeedotrema* (Morphogroups: DIGE10, DIGE13, DIGE20, META01) and *Caudotestis* (Morphogroups: DIGE01, META08) as well as an unidentified cercaria (Morphogroup: CERC01). (b) Phylogenetic trees resulting from maximum likelihood analysis of the 435 bp partial ITS2 rDNA alignment including individuals of only *Biospeedotrema* and *Caudotestis*, as this clade had the most subtle differences. In the sample names, the parasite morphogroup ID is listed first, followed by an individual number if multiple parasite individuals were sequenced within a host individual. The morphogroup prefix DIGE indicates the adult life stage, META indicates the metacercaria life stage, SPOR indicates the sporocyst life stage, and CERC indicates the cercaria life stage. Next, the host species is listed followed by the host individual number. Some morphogroups split into two clusters, indicating hidden diversity or error in identification. These split clusters are denoted with "a" and "b", also referenced in Table S1. Support values were generated with 1,000 bootstraps. The scale bar indicates the expected number of substitutions per site.

Discussion

Given the observed genetic variation among *Biospeedotrema* morphogroups, we elected to keep them separated in our study although they may ultimately prove to be a single species. The amount of genetic variation is consistent with variation seen within species in other studies (usually accepted as 0.1–1.5%) (Nolan & Cribb 2005), however, the variation in both morphology and genetics leads us to believe these might constitute separate taxa early in speciation. In a meta-analysis, for example, 44 studies analyzed the ITS2 fragment and only 16 (36%) found any intraspecific variation that ranged from 0.3–3.5% (Nolan & Cribb 2005). In our study, the *Biospeedotrema* and *Caudotestis* morphogroups clearly separated into their distinct genera morphologically despite varying only 0.2–1.0% in the 435 bp fragment. If the morphogroups with very similar ITS sequences do eventually get lumped into a single species, our paper would include: (1) one *Biospeedotrema* species that matches to the metacercaria META01, (2) one *Caudotestis* species that matches to the metacercaria META08, (3) one *Neolebouria* species that quite definitively matches SPOR01, and (4) a single cercaria morphotype unmatched to other life stages.

LITERATURE CITED

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