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Boergesenia parvula sp. nov. (Siphonocladales, Chlorophyta) from the Tropical Western Atlantic

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Abstract – *Boergesenia parvula* sp. nov. is described from shallow water habitats in Puerto Rico and represents the smallest species of the genus, reaching only 20 mm in height. Recognition of the new entity is supported by molecular sequences of the 18S rRNA gene. Molecular divergence between *B. forbesii* and the newly described species is 3.4%.

18S rDNA sequences / *Boergesenia parvula* sp. nov. / Caribbean Sea / Chlorophyta / Siphonocladales / Puerto Rico / Taxonomy

Résumé – *Boergesenia parvula* sp. nov. est décrit. Cette espèce vit dans des habitats peu profonds de Puerto Rico et représente la plus petite espèce du genre ne dépassant guère 20 mm de haut. La reconnaissance de ce nouveau taxon est basée sur la séquence moléculaire du gène codant l'ARN 18S. La divergence moléculaire entre *B. forbesii* et la nouvelle espèce présentée ici est de 3,4 %.

***Boergesenia parvula* sp. nov. / Chlorophyta / Mer des Caraïbes / Puerto Rico / Siphonocladales / Séquences 18S rDNA / Taxonomie**

INTRODUCTION

Boergesenia has had an interesting taxonomic history as elucidated by Silva *et al.* (1996). The genus is based on *Valonia forbesii* Harvey and was originally distributed in Harvey's Ceylon exsiccata (Harvey, 1857: No. 75) without a description. Several years thereafter, Harvey (1860) published a short Latin account which validated the new species. Subsequently, both J. Feldmann (1938) and Iyengar (1938a,b) independently transferred *V. forbesii* to new genera, *Boergesenia* and *Pseudovalonia* respectively. Silva *et al.* (1996) discussed the validity of both genus names and concluded that *Boergesenia* was fully validated

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on 16 May (J. Feldmann, 1938) while *Pseudovalonia* was not validated until June (Iyengar, 1938b). Therefore, *Boergesenia forbesii* (Harvey) J. Feldmann is now recognized as the binomial for the species. Harvey's specimen (No. 75) from Ceylon was formerly considered to be the type (in TCD); however, Harvey (1860) provided the type location as "Loo Choo Islands. (Also Ceylon)" with the Latin description. The Loo Choo Islands are now called the Ryukyu Islands (southern Japan) in the East China Sea, and Lipkin and Silva (2002) indicated that the type in TCD was from Ryukyu-retto, Japan and collected by C.Wright. They (*op. cit.*) illustrated a highly atypical branched specimen.

Boergesenia forbesii has been broadly reported from the tropical Pacific Ocean including the Red Sea and Indian Ocean (Børgesen, 1936; plus see references in Silva *et al.*, 1996), Southeast Asia (Silva *et al.*, 1987), Australia (Huisman, 2000), Marshall Islands (Taylor, 1950) and Palau (Ohba *et al.*, 2007). A second species, *B. magna* Kraft (2007) was subsequently added with a type location in the southern Great Barrier Reef, Australia.

The genus was originally characterized by possessing unbranched pyriform or clavate vesicles which form incurved rosette-like clusters although *Boergesenia magna* forms solitary vesicles. Annular constrictions are present on the tapered proximal vesicle portions of both species (Enomoto & Hirose, 1972; Kraft, 2007; Guiry & Guiry, 2010). Ballantine *et al.* (2009) reported occurrence of a diminutive *Boergesenia* species from very shallow-water habitats in Puerto Rico. Recognizing that the substantially smaller size in their specimens in addition to the substantial geographic separation might indicate a separate species, they elected to treat their specimens as *B. forbesii* pending molecular comparison. We have since sequenced specimens from both Japan and Puerto Rico and on this basis we describe herein a new *Boergesenia* species. More recently we have discovered what we initially thought was a fourth *Boergesenia* species. However, on the basis of molecular and morphological comparison, we conclude that the latter entity represents an Atlantic record of *B. forbesii*.

MATERIALS AND METHODS

Specimens were collected by snorkeling at Magueyes Island (La Parguera), the Condado Lagoon (San Juan), Puerto Rico and from Ishigaki Island, Ryukyu Islands, southern Japan. Some of these were preserved in 10% formalin/seawater for morphological examination and some desiccated in silica gel for DNA analysis. Photomicrographs were taken with a SPOT RE digital camera through an Olympus BMAX light microscope. Paratype specimens have been deposited in MICH, MSM and US. Herbarium abbreviations follow Holmgren *et al.* (1990), and authority designations are in accordance with Brummitt and Powell (1992).

DNA extraction, amplification, sequencing and phylogenetic reconstruction

Total genomic DNA from algal specimens (Table 1) was isolated using the protocol described by Ballantine and Lozada-Troche (2008). The 18S rRNA gene fragments were generated using primer combinations SR1-SS11H and SSU897-18SC2 (Leliaert *et al.*, 2007). PCR amplifications were carried out in 200 µl

Table 1. 18S rDNA sequences used in the phylogenetic analyses.

Species	*Source*	*GenBank Accession Number*	*References*
Boergesenia forbesii	GenBank	AM498746	Leliaert *et al.*, 2007
B. forbesii	GenBank	AF510164	Unpublished
B. forbesii (CLT-301)	Ishigaki Island, Japan	HQ173706	This paper
B. forbesii (DLB-8175)	San Juan, Puerto Rico	HQ173705	This paper
B. parvula (DLB-7546)	La Porguara, Puerto Rico	HQ173703	This paper
B. parvula (DLB-7809)	La Porguara, Puerto Rico	HQ173704	This paper
Cladophora aokii	GenBank	AM498747	Leliaert *et al.*, 2007
C. coelothrix	GenBank	AM498749	Leliaert *et al.*, 2007
C. coelothrix	GenBank	Z35315	Bakker *et al.*, 1994
C. prolifera	GenBank	AM498750	Leliaert *et al.*, 2007
C. prolifera	GenBank	Z35422	Bakker *et al.*, 1994
Cladophoropsis membranacea	GenBank	AF510150	Unpublished
C. membranacea	GenBank	AF510151	Unpublished
C. membranacea	GenBank	AF510152	Unpublished
C. membranacea	GenBank	AF510153	Unpublished
C.membranacea	GenBank	AF510160	Unpublished
C. sundanensis	GenBank	AF510154	Unpublished
Chaetomorpha crassa	GenBank	AB062701	Hanyuda *et al.*, 2002
Dictyosphaeria cavernosa	GenBank	AM498755	Leliaert *et al.*, 2007
D. cavernosa	GenBank	AM498756	Leliaert *et al.*, 2007
Ernodermis verticillata	GenBank	AM498757	Leliaert *et al.*, 2007
E. verticillata	GenBank	AM498758	Leliaert *et al.*, 2007
E. verticillata	GenBank	Z35321	Bakker *et al.*, 1994
Siphonocladus tropicus	GenBank	AM498761	Leliaert *et al.*, 2007
S. tropicus	GenBank	Z35313	Bakker *et al.*, 1994
Valonia aegagropila	GenBank	AM498762	Leliaert *et al.*, 2007
V. fastigiata	GenBank	AM498763	Leliaert *et al.*, 2007
V. utricularis	GenBank	Z35323	Bakker *et al.*, 1994
Valoniopsis pachynema	GenBank	AM498764	Leliaert *et al.*, 2007
V. pachynema	GenBank	AM498765	Leliaert *et al.*, 2007
Ventricaria ventricosa	GenBank	AM502590	Leliaert *et al.*, 2007

microtubes, with a total volume of 50 µl containing 2.5 µM of nucleotide mix, 20-30 pmol of each primer, 2.5 Units of Taq Polymerase (Eppendorf Hotmaster Taq or Promega, Madison, USA) and 10X reaction buffer. Amplifications were performed in an Eppendorf Gradient Mastercycler using an initial 3 min denaturation at 94°C followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, 1 min 30 sec extension at 72°C, and a final extension of 3 min at 72°C. PCR amplifications were verified using a 1% agarose gel stained with ethidium bromide (10 mg/ml) under UV light (Sambrook *et al.*, 1989). Length of amplified fragments was

determined by comparison with the migration of Lambda Hind III plus marker (Lambda Biotech Inc.). PCR products were purified for sequencing reactions using the Perfectprep® Gel Cleanup Kit (Eppendorf).

The sense and reverse strands of the amplicons were sequenced using the Applied Biosystems Big Dye™ Terminator v3.1 and ABI 3130 xl genetic analyzer. The sequencing reaction consisted of reaction reagent, Big Dye terminator reaction buffer, 50-70 ng of PCR product, 3 pmol of the forward or reverse PCR primers and dH_2O in a total volume of 10 µl. Sequencing was carried out at the Genotyping facility, University of Puerto Rico, Río Piedras. DNA sequences were edited with Sequencher 4.6 (Gene Codes Corp, Ann Arbor, MI, USA) and aligned and compared using CLUSTALX 2.0.11 (Thompson *et al*., 1997).

The optimal model determined by Modeltest 3.7 (Posada and Crandall, 1998) used as input for NJ and ML analysis calculated by the Akaike information criterion (AIC) (Akaike, 1974) for 18S alignment was the TrN + I + G evolutionary model (Tamura Nei, model + Proportion of Invariable Sites + Gamma distribution) (Tamura & Nei, 1993). The determined nucleotide frequencies were: A = 0.2636, C = 0.2182, G = 0.2724, T = 0.2458. The substitution rate matrix used in the analysis was A-C = 1.0000, A-G = 1.9823, A-T = 1.0000, C-G = 1.0000, C-T = 3.5746, G-T = 1.0000. The proportion of invariable sites was 0.4795, rates for variable sites follow a gamma distribution with shape parameter = 0.8200. The phylogenetic reconstruction for the new species was assembled using the maximum parsimony (MP), maximum likelihood (ML), and neighbor-joining (NJ) algorithms as implemented in PAUP* v 4.1b10 (Swofford, 2002). Bayesian Inference (BI) was performed with MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001). The robustness of the phylogenetic reconstructions was determined by bootstrapping the data set (Felsenstein, 1985) 2,000 times for MP and NJ and 100 times for ML. Bayesian analysis was conducted using the General Time Reversal (GTR) evolutionary model running 1,000,000 generations (Lanave *et al.*, 1984, Rodriguez *et al.,* 1990). Trees were sampled every 100 generations, and log-likelihood scores stabilized (X-Y scatter plot) at approximately 5,500 generations. The first 6,500 of the 10,000 trees were discarded as burn-in.

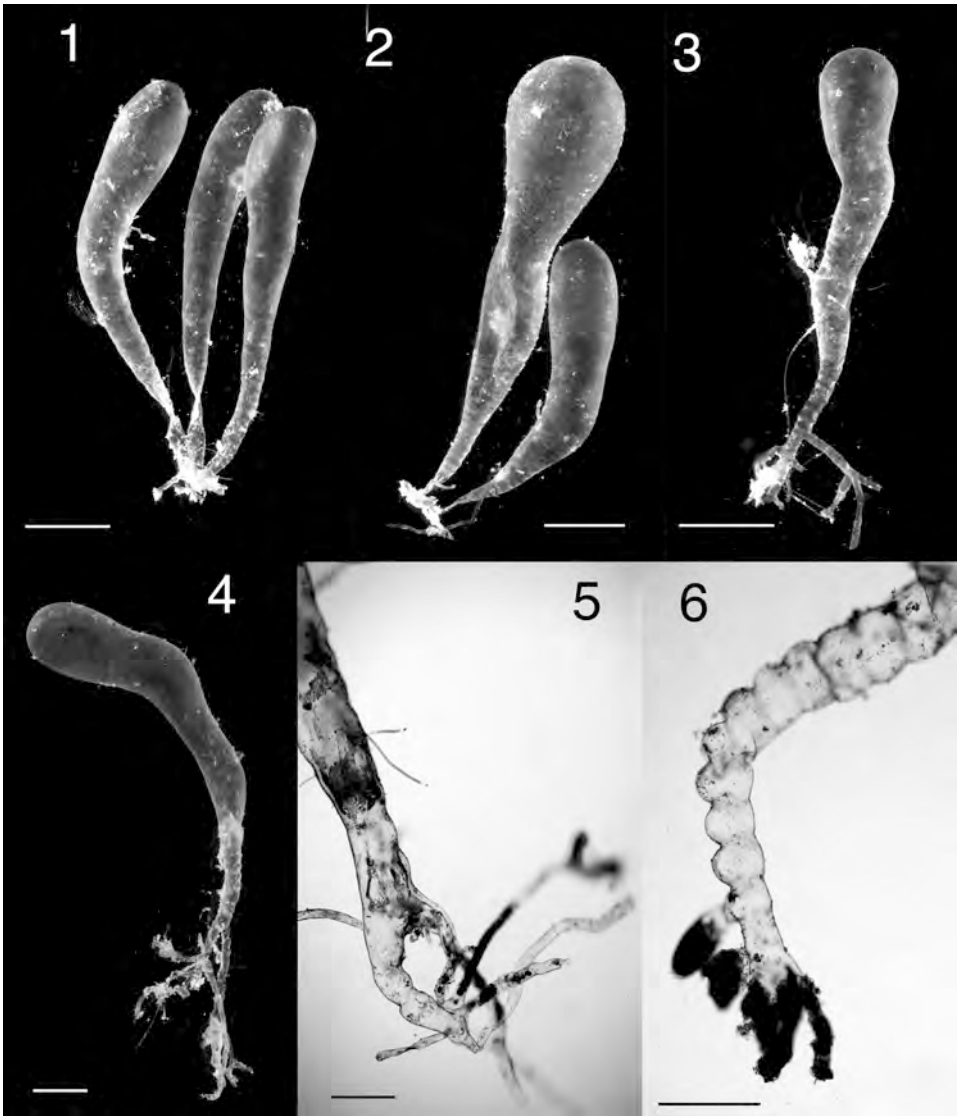
RESULTS

Boergesenia parvula D.L.Ballant., Ohba *et* Lozada-Troche sp. nov. Figs 1-6

Diagnosis

Plantae vesiculares, vesiculis fasiculatis, 2-3 usque ad multae per fasiculam; vesiculae valde irregulariter formatae, paene cylindricae usque ad expansas supra; vesiculae singulae usque ad 20 mm longae, 0.3-0.4 mm diametro basibus annulatis et usque ad 2.6 mm latae subapicaliter; fasiculi vesicularum rhizoideis communibus conligati, 60-70 µm diametro; reproductio ignota.

Holotype: *D.L.B.8170*, Magueyes Island, La Parguera, Puerto Rico (17°58.225 N, 67°02.777 W) on stones beneath *Rhizophora mangle* L., < 0.5 m deep, coll. David L. Ballantine and Hector Ruiz, 15.vi.2010 (US Alg. Coll.-217084).



Figs 1-6. *Boergesenia parvula* sp. nov. (all *DLB8170*). **1.** Photograph of the living holotype showing a cluster of three vesicles. Scale bar = 2.0 mm. **2.** A cluster of two vesicles, note annular constrictions on vesicle on left. Scale bar = 2.0 mm. **3.** A single vesicle showing rhizoids issuing from different levels of the annularly constricted vesicle base. Scale bar = 2.0 mm. **4.** A single vesicle showing clusters of rhizoids issuing from different levels of annularly constricted plant base. Scale bar = 1.0 mm. **5.** Basal portion of vesicle showing multiple rhizoids produced from near the vesicle base. Scale bar = 100 µm. **6.** Basal plant portion showing rhizoids produced at different levels. Scale bar = 500 µm.

Paratypes: H.0.10001,2 Magueyes Island, La Parguera on rubble beneath *Rhizophora mangle* and on marine rail, < 0.5 m, coll. *H. Ohba*, 27.vii.2008; *D.L.B.7546, ibid*., on marine rail, coll. *Hector Ruiz*, 5.viii.2008. Deposited in MSM, MICH and US.

Etymology: The specific epithet refers to the fact that the new species is substantially smaller than the previously known *Boergesenia* species.

Boergesenia parvula has only been collected in very shallow water. It was first collected on a marine rail at 10 to 20 cm depth on the west side of Magueyes Island, La Parguera. Subsequently it was collected at the same depth on rubble, stones and pieces of wood on a silty bottom beneath branches of the mangrove *Rhizophora mangle* L. approximately 100 m north from its original collection site. Plants are vesicular with vesicles occurring in clusters of two or three to many (Figs 1, 2). The vesicles are somewhat irregular in shape, being nearly cylindrical to expanded above. They also range from being arcuate to nearly straight. Individual vesicles measure to (10) 12-18 (20) mm long and 0.3 to 0.4 mm diameter at their bases and to 2.6 mm at their broadest subapically. The vesicle bases are variably annularly constricted, the annulations being distinct in some plants (Figs 2, 3, 4, 6) and barely discernible in others (Figs 1, 5). Vesicles are rarely branched, occasionally giving rise to another vesicle laterally from near the base. The clusters of vesicles are held together by common entangled rhizoids, 60 to 70 μm in diameter. Rhizoids issue from both the base of vesicles as well as from the lower annulated region (Figs 3-6). The rhizoids terminate in digital pads where they attach to the substratum.

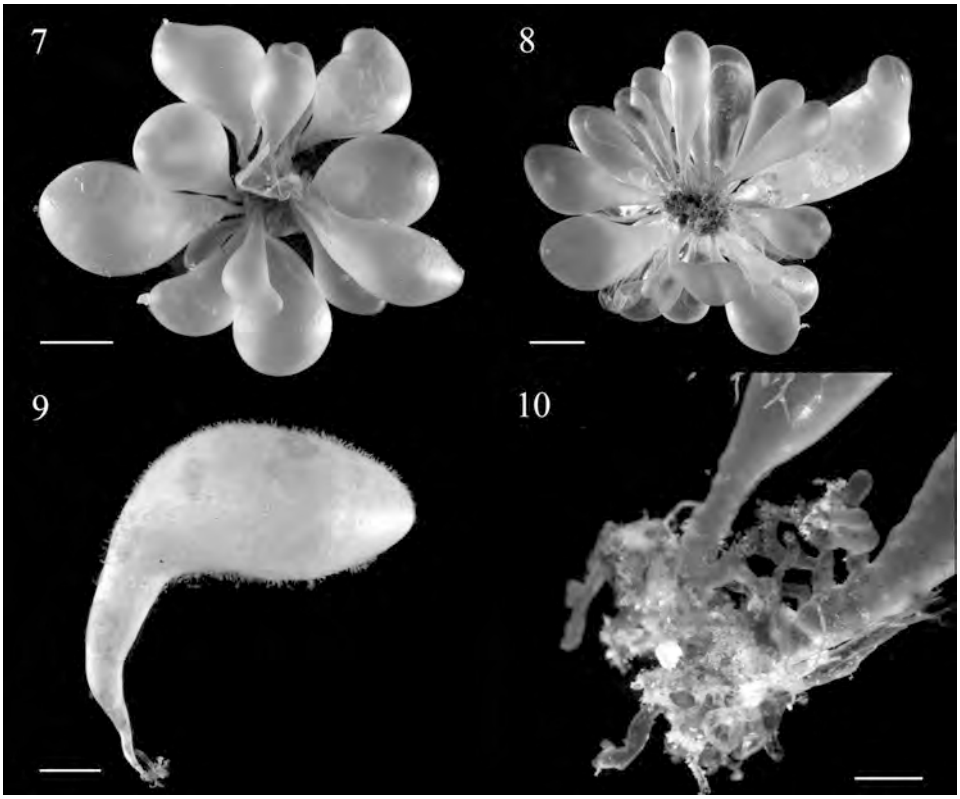
***Boergesenia forbesii* (Harvey) J. Feldmann** **Figs 7-10**

Collections: *D.L.B.8175*, Condado Lagoon, San Juan, Puerto Rico (18°27.766 N, 66°06.202 W), 0.2 m depth, Coll. H.R., 19.vi.2010 (US Alg. Coll.-217083); *D.L.B.8237*, Condado Lagoon, 0.2-0.5 m, Coll. H.R., 26.viii.2010.

Plants occur in clusters of up to 15 conspicuous large vesicles (Figs 7, 8). The vesicles are frequently arched (Fig. 9), reaching 6.5 cm in height. The vesicles are narrow near their base, to 0.7 mm diameter, and expanded above, to 13 mm (Figs 7-9). Vesicles are normally inconspicuously annulate at their bases (Fig. 10) although occasionally smooth (Fig. 9). The vesicles produce abundant rhizoids from their lower stipes (Fig. 10). The rhizoids form a conspicuous, expanded and highly entangled mat and young vesicles are abundantly produced from the rhizoids. At the lower surface of the mat, rhizoids give rise to terminal digitate attachments.

Phylogenetic analysis

Four new *Boergesenia* DNA sequences (1706 bp in length) were compared with 27 siphonocladalean sequences obtained from Genbank. *Chaetomorpha crassa* was chosen as outgroup. For MP analysis 160 characters were parsimony-informative, tree length was 474, consistency index (CI) = 0.76, and retention index (RI) = 0.82. The phylogram indicates that *Boergesenia* is included within a clade comprised of siphonocladalean genera (Fig. 11). Within the Siphonocladales clade, *Boergesenia* is distinct from *Siphonocladus*, *Ernodesmis*, *Dictyosphaeria*, *Valonia* and *Cladophoropsis* (*Boodlea* complex). *Boergesenia parvula* is well separated from *B. forbesii* in the phylogram (Fig. 11) supporting the erection of the new species. Sequence divergence between *B. parvula* and *B. forbesii* was 3.4% as calculated by PAUP using p-uncorrected distances (Swofford, 2002). The average intraspecific divergence of *B. forbesii* specimens included in the analysis was 0.69%.



Figs 7-10. *Boergesenia forbesii* from Puerto Rico. **7.** Habit of a large colony (*DLB8175*). Scale bar = 1.0 cm. **8.** Habit of a large colony, note the dark central region representing a dense rhizoidal pad (*DLB8237*). Scale bar = 1.0 cm. **9.** A single vesicle teased from a colony. The basal region lacks annular constrictions (*DLB8175*). Scale bar = 1.0 cm. Fig. 10. The base of two vesicles showing the rhizoidal mat with abundant closely adhering rhizoids (*DLB8237*). Scale bar = 2.0 mm.

DISCUSSION

The morphology of *Boergesenia parvula* is typical of the genus in that plants sometimes form arcuate vesicles, some of which distally expand from a narrow annulate base. In an earlier publication (Ballantine *et al.*, 2009), the species was referred to *B. forbesii* on the basis of overall morphological similarity despite the fact that substantial differences in size existed. *Boergesenia parvula* has maximum vesicle dimensions of 20 mm in length and 2.6 mm in width. *Boergesenia forbesii* is substantially larger with reported measurements to 5 (-8) cm in length, and to 2 cm in diameter (Taylor, 1950; Tseng, 1984, Littler & Littler, 2003; Ohba *et al*., 2007). *Boergesenia magna* also differs in its much larger size to 10.5 cm tall and to 3.5 cm in breadth in addition to its solitary habit (Kraft, 2007).

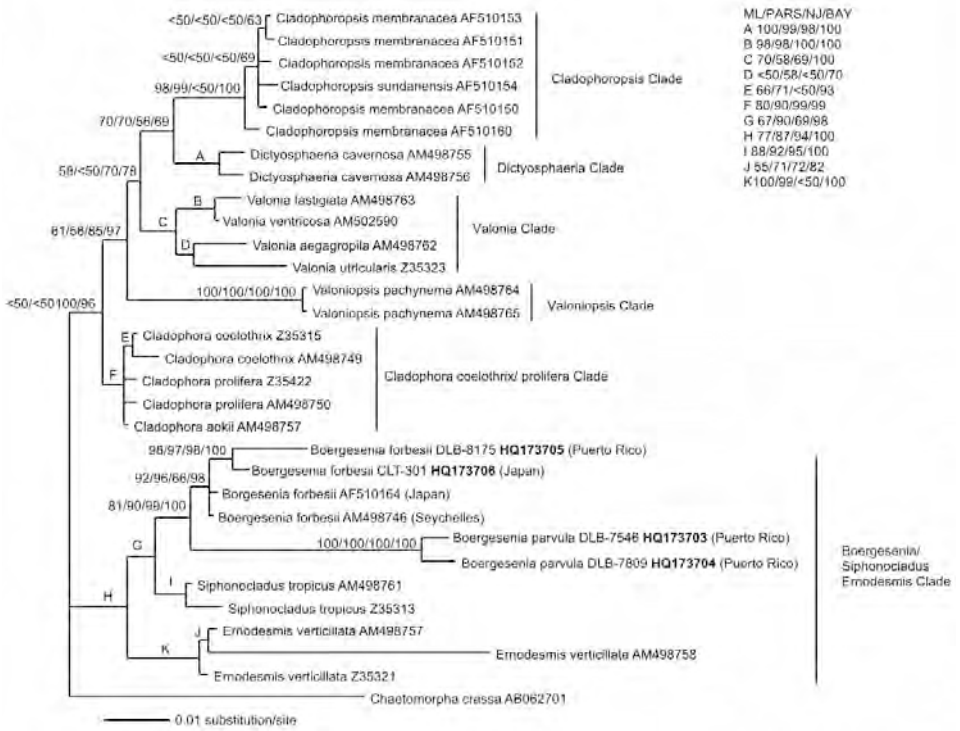


Fig. 11. Bayesian Inference phylogenetic tree for selected members of the Siphonocladales using the Small subunit ribosomal DNA (18S rDNA) gene. Bootstrap proportions and posterior probabilities are shown on top of the branches. Left to Right (Maximum Likelihood; 100 replicates), (Maximum Parsimony; 2000 replicates), (Neighbor Joining; 2000 replicates), and (Bayesian Posterior Probabilities; 1,000,000 generations). Accession numbers in bold represent sequences new to this study.

Our phylogram agrees in overall topology with previous Siphonocladales studies where *Boergesenia* forms a distinct clade with *Siphonocladus* and *Ernodesmis* inferred from partial LSU rRNA gene sequences (Leliaert *et al*., 2003, 2007). Erection of *B. parvula* as a new species within the genus is supported by both morphological differences and molecular divergence. While Leliaert *et al.* (2007) found an LSU rDNA sequence divergence of only 0.2 percent for three widely divergent *B. forbesii* populations, the degree of molecular divergence between *Boergesenia forbesii* collections from Puerto Rico and Japan indicates that further molecular studies of more variable loci, such as LSU rDNA or ITS may reveal subspecific diversification or presence of cryptic speciation. For example, ITS sequences revealed at least 13 distinct species in the *Boodlea* complex within the Siphonocladales (Leliaert *et al*., 2009).

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