Taxonomic and molecular phylogenetic studies of the genus *Colpomenia* (Scytosiphonaceae, Phaeophyceae)

【褐藻フクロノリ属（カヤモノリ科）の分類学的および分子系統学的研究】

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ABSTRACT

The classification of the brown algal family Scytociphonaceae still remains problematic because of their morphological plasticity and also the molecular phylogenetic inconsistencies among its genera. With the help of several phycologists, some of the issues have been addressed, but the problem is still there especially in the genus *Colpomenia*.

I attempted in this study to resolve taxonomic problems within this genus and its species by combining multi-gene molecular data with morphological observations and life histories. I assessed the taxonomy of all known globular species, using *Colpomenia* samples from Japan, Russia, Australia, and Portugal. Three molecular markers were used for phylogenetic analyses: mitochondrial *cox*3 gene and plastidal *rbc*L and *psa*A genes. In all *cox*3, *rbc*L, and *psa*A trees reconstructed, this genus revealed four major evolutionary lineages (Lineages I–IV). In addition, a new species has been discovered while conducting this research.

Colpomenia borea is characterized by its small globular to ovoidal thalli and thin thallus membrane and is epiphytic on the brown alga *Stephanocystis*. This species was found in the Pacific coast of Hokkaido, Japan, and Magadan, Far East Russia which are the coldest regions in the distributional range of *Colpomenia*.

This study revealed independence of each of Lineages I–IV, suggesting polyphyly of the genus *Colpomenia*. Lineage I consisted of *Colpomenia sinuosa* which is the generitype of the genus. Lineage II consisted of *C*. *claytoniae*, *C*. *expansa*, *C*. *peregrina*, and *C*. *borea*, which have globose thalli. Since this Lineage

was distantly related to the generitype *C*. *sinuosa*, a new genus name should be proposed for the Lineage II. Lineages III and IV were composed of *C*. *ecuticulata* and *C*. *ramosa*, respectively. These Lineages were also distantly related to *C*. *sinuosa* in *cox*3 analyses.

In *cox*3 trees, highly supported subclades were formed in *C*. *sinuosa* and *C*. *claytoniae*, suggesting multiple species within those species. *Colpomenia peregrina* also showed large intraspecific sequence differences. Four DNA-based species delimitation analyses (ABGD, ASAP, PTP, bPTP) were also conducted for *Colpomenia* species, using *cox*3 sequences. Results of ABGD and ASAP were similar, suggesting two or four species within *C*. *sinuosa* and a single species for *C*. *claytoniae* and *C*. *peregrina*. In contrast, PTP and bPTP yielded nine species within *C*. *sinuosa* and five species within *C*. *claytoniae* while a single species for *C*. *peregrina*. Species boundaries that corresponded among all species delimitation analyses supported the current classification except for *C*. *sinuosa* which was divided into two by the boundary. *Colpomenia sinuosa* could include at least two species.

Because globose *Colpomenia* are similar in gross morphology, they may have been misidentified. In this study, molecular identification of *Colpomenia* species from Japan was conducted, and their more accurate distributional ranges were revealed in Japan.

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CONTENTS

Chapter 1

GENERAL INTRODUCTION

The algae, consisting of numerous organisms with extensive branches in the eukaryotic lineage, underwent evolution approximately 1.5 billion years ago. (Douzery *et al*. 2004; Parfrey *et al*. 2011; Gibson *et al*. 2017; Blaby-Haas & Merchant 2019). Algae do not have a single common ancestor and are spread throughout eukaryotic lineages; most algae are related to endosymbiosis that resulted in the transfer of plastids and genes to diverse eukaryotic hosts and thus created lineages of algae (Blaby-Haas & Merchant 2019).

The brown algae, class Phaeophyceae Kjellman 1891, are one of few eukaryotic lineages to have evolved complex multicellularity, displaying great diversity of morphology and physiology across species, and probably evolved around 260 million years ago during the Permian Period from an organism called Phaeothamniophyceae, which have motile cells same as Phaeophyceae, but lack unilocular and plurilocular sporangia of the Phaeophyceae (Fritsch 1935; Bailey *et al*. 1998; Charrier *et al*. 2008; Brown 2010; Knoll 2011; Yang *et al*. 2012;

Cock *et al*. 2014; Liu *et al*. 2014; Kawai *et al*. 2015; Lee 2018; Bringloe *et al*. 2020). They belong within the stramenopiles that had chloroplasts originated from secondary endosymbiosis wherein a red alga was engulfed by a non-photosynthetic protist (Keeling 2010; Liu *et al*. 2015a). Like other multicellular groups (metazoans, fungi and green plants), brown algae have several key features that enabled them to thrive as macroscopic organisms: cell-to-cell adhesion and communication, tissue differentiation, internal transport of sugars, and the capacity for three-dimensional growth (Fritsch 1935; Schmitz & Srivastava 1976; Kloareg & Quatrano 1988; Charrier *et al*. 2008; Cock *et al*. 2010, 2014; Deniaud-Bouët *et al*. 2014; Starko & Martone 2016). Phaeophyceae was first classified by Kylin in 1933 mainly by the life history patterns, growth modes (diffuse growth, apical growth, trichothallic growth or meristematic growth), thallus construction (filamentous, pseudoparenchymatous or parenchymatous) and their way of sexual reproduction (-isogamy, anisogamy or oogamy) (Papenfuss 1951a, b; Scagel 1966; Wynne & Loiseaux 1976; Bold & Wynne 1978; Van den Hoek & Jahns 1978; Womersley 1987; Van den Hoek *et al*. 1995; Rousseau & Reviers 1999; Dy 2019).

The brown algae as of today is comprised of approximately 2000 described species. They are a group of morphologically diverse organisms, varying from microscopic filaments to huge or tall macroscopic seaweeds like kelps (Siemer *et al*. 1998). Brown algae are one of major photosynthetic producers of organic carbon on rocky intertidal shores worldwide (Liu & Pang 2015a). This class exhibits, hence, the name, brown to golden-brown to dark brown in color or can be sometimes olive green in color which come came from their chromatophores of an accessory carotenoid pigment, fucoxanthin and various phaeophycean

tannins in some species, and their cell walls are composed of cellulose giving them mucopolysaccharide alginic acid which can harvest in adequate quantities for commercial purposes (Smith 1951; Wehr 2003). Species of brown algae can thrive almost solely in marine water, about 1% are in known freshwater and some in brackish water habitats, and they have greater diversity in cold, temperate to tropical waters worldwide (Wilce 1966; Dop 1979; West & Kraft 1996; Draisma 2002; Wehr 2003; Liu & Pang 2015b; Dy 2019).

The brown algae systematics in general (i.e., Heterokonta, Oehrophyta, Phaeophyceae) has had a complicated history where the first evolutionary classifications of the gradualist views of the authors, Kylin (1933), Papenfuss (1951a, 1951b), Scagel (1966), Wynne & Loiseaux (1976), analyzed a small number of morpho-anatomical and reproductive features, such as the mode of growth, thallus structure, and -gamy types and life history (Reviers & Rousseau 1999; Silberfeld *et al*. 2014). Which is why the class Phaeophyceae have continued to challenge systematists and phycologists over the past century (Draisma *et al*. 2001, McDevit & Saunders 2017; Dy 2019).

In its earliest time of the research on brown algae, phylogenetic studies were limited by the coarse resolution of chosen markers, i.e., 18S (Tan & Druehl 1993, Bringloe *et al*. 2020). However, despite using remarkable molecular techniques for the past decades, studies in Phaeophyceae failed to provide a clear classification because of multiple contradictions. Due to unexpected results of their vast range and plasticity in morphological forms (Silberfeld *et al*. 2014; Bringloe *et al*. 2020) such as phenotypic plasticity and lack of detailed diagnostic characters in some groups, delimitation of species using conventional methods is

often problematic and has led to false or inaccurate taxonomic assignments (Tellier *et al*. 2009; Tronholm *et al*. 2010; Geoffroy *et al*. 2015).

With continuous effort, progress has been made dramatically which increased our knowledge of the brown algal systematics by combining multiple markers, time calibrated phylogenies, and more recent genome-scale datasets in some brown algal groups (Silberfeld *et al*. 2010; Martin & Zuccarello 2012; Jackson *et al*. 2017; Starko *et al*. 2019; Yip *et al*. 2020; Bringloe *et al*. 2020). Significant amount and accumulation of sequence data through time is now available to molecular systematists for inferring the evolutionary history, identification of species and to provide multiple gene and genome datasets that can be used to reconstruct more robust trees (Chase *et al*. 1993; Gadagkar *et al*. 2005) and those were essential to expand our complete understanding of the class Phaeophyceae (Silberfeld *et al*. 2014; Liu & Pang 2015b). In addition, these resulted to sudden alterations on how we view these brown algal relationships and the traits evolution (Bringloe *et al*. 2020).

Among many orders of the Phaeophyceae is the order Ectocarpales Bessey 1907. Rousseau and Reviers (1999) have analyzed the Ectocarpales' lengthy and muddled circumscription in detail. They made great progress by revisiting the idea of the Ectocarpales with the help of multigene phylogenies, and they have now expanded the ordinal notion to encompass all brown algae that bear plastids with stalked pyriform pyrenoids; and as a result, the Ectocarpales *sensu stricto* were incorporated with the Chordariales Setchell *et* Gardner 1925, Dictyosiphonales Setchell *et* Gardner 1925, and Scytosiphonales J. Feldmann 1949 based on priority (Santiañez 2018), excluding the family Ralfsiales and taxa with stellate plastids (Cho *et al*. 2006).

In 2001, a new system was proposed by Peter and Ramirez in which the order Ectocarpales consisted of five families: Acinetosporaceae G.Hamel ex J.Feldmann 1937, Adenocystaceae F.Rousseau, B.de Reviers, M.-C.Leclerc, A.Asensi, & R.Delépine 2000, Chordariaceae Greville 1830, Ectocarpaceae C.Agardh 1828, and Scytosiphonaceae Farlow 1881 (Peter & Ramirez 2001; Cho *et al*. 2006). An updated classification was followed years after and the Petrospongiaceae Racault *et al*. was added to the family. Currently, the order Ectocarpales consists of six families.

In the order Ectocarpales, among those families taxonomically accepted above, is the family Scytosiphonaceae Farlow 1881. Members of this family are distributed from intertidal to subtidal habitats that occur in tropical, warm and cold waters worldwide (Lee *et al*. 2014b). This family has long been notoriously problematic, complex, and confusing throughout the years in its genus and species relationships, both morphological and phylogenetical aspects. Earliest phylogenetic studies have revealed that this family exhibited a high degree of paraphyly and polyphyly in several genera (Kogame *et al*. 1999; Cho *et al*. 2001, 2006; Santiañez & Kogame 2022).

Classification in the Scytociphonaceae family still has problems in their generic characteristics, resulting to an ambiguous delineation in which it made more difficult due to their broad morphological plasticity among species known in this family (Kogame *et al*. 1999; Santiañez 2018). Based on the dissertation of Santiañez (2018), I listed brief morphological characteristics of some genera representatives under this family:

***Hydroclathrus* Bory 1825:** net-like and spreading, the most distinct morphology in this family. But the young thalli of this genus could be mistakenly as a *Colpomenia* species due to the saccate or sometimes perforated.

***Rosenvingea* Børgesen 1914:** are distinguished by their erect, dichotomous, or alternately branched, cylindrical to somewhat compressed, hollow thalli. Most species, branches are free, in some co-adhering. However, this genus is currently problematic due to their branching pattern. There were studies conducted before, but only molecular analyses were done and there were no detailed descriptions of morphology. Thus, this genus needs scrutiny to further delimit them.

***Iyengaria* Børgesen 1939:** was established based on previously assigned to *Rosenvingea stellata* and *Colpomenia stellata*. But it was classified as monotypic based on semiglobular thallus provided with more or less solid, conical projections giving the plant a semi-stellate appearance by Børgesen (1939). Additional reassessment has been suggested should be done in this genus.

***Colpomenia* (Endlicher) Derbès & Solier 1851:** saccate, globular to irregularly convoluted, some branched or irregular protrusions that overlap with other genus such as: *Iyengaria*, *Rosenvingea*, and *Scytosiphon*.

***Chnoospora* J.Agardh 1847:** has an erect to decumbent and branching or inter-adhesive and are distinguished from *Rosenvingea* in possessing solid thalli. Some species here before were not monophyletic, and thus it led to establishing new genus, *Pseudochnoospora*.

***Scytosiphon* C.Agardh 1820, nom. cons.:** distinguished as compressed or cylindrical to flattened, erect thalli and hollow to partially hollow or unbranched, having constrictions or not and having ascocysts or not. Sometimes these species are difficult to identify when in field, thus, to confirm them is through genetic sequencing. However, from observed gene trees from this study and from previous studies, this genus was not recovered as monophyletic.

***Myelophycus* Kjellman 1893:** has caespitose, upright and simple, solid (when young) to hollow (when old) and isomorphic and is morphologically similar to the monotypic genus *Melanosiphon*. However, Tanaka and Chihara (1984) made a detailed review of both genera and they suggested that these are congeneric based on widely overlapping characters. While *Melanosiphon* is distinguished from *Myelophycus* in having paraphyses with longitudinal septa.

***Petalonia* Derbès & Solier 1850, nom. cons.:** has leaf-like and flattened thalli, that are hollow to partially hollow to solid and has entangled rhizoidal filaments in its medulla.

Systematic problems in the Scytosiphonaceae, such as taxonomic inconsistencies, classification, and phylogeny still exist today. Recent work done by several phycologists has been an eye-opener to everyone. First, wherein Kogame (1994) introduced the combination of morphology and life history characteristics to delineate different genus and species. Second by Kogame *et al*. (1999), both morphology and life history characters integrate with molecular phylogeny. And lastly, Santiañez (2018) provided a framework where all these

should combine and be a necessity for clarification and distinction at the generic level in the family Scytosiphonaceae.

To at least correct the classification of the family Scytosiphonaceae, recent work and effort by Santiañez *et al*. (2018); Santiañez (2023), he proposed two tribes: the Hydroclathreae and the Scytosiphoneae. Based on their study, the Hydroclathreae tribe was composed of species with morphologies ranging from erected to spreading, saccate to branching and some possess anastomosing to varying degrees of either hollow or firm construction. While members of Scytosiphoneae tribe have hallowed, partially hallow or firm with an erect, elongated, and terete to flattened thalli. In addition to these tribes, detailed reassessment of different scytosiphonacean taxa using molecular phylogenies and morphologies has led to the separation and transfer of several species into new genera such as *Dactylosiphon* Santiañez, K.M.Lee, S.M.Boo & Kogame 2018, *Planosiphon* McDevit & G.W.Saunders 2017, and *Pseudochnoospora* Santiañez, G.Y.Cho & Kogame 2018 (McDevit & Saunders 2017; Santiañez & Kogame 2022).

THE GENUS *COLPOMENIA*

Out of all current genera of the tribe *Hydroclathreae*, the genus *Colpomenia* (Endlicher) Derbès *et* Solier 1851 is still an interesting genus. This genus are widely known in temperate to tropical in mid or low intertidal zones waters around the world (Boo *et al*. 2011; Song *et al*. 2019). These species can be recognized by their habit: attached on the rock or some *Sargassum* species to

even oysters, thus they were monikered as “oyster thieves” causing substantial damage to the oyster industry in which when they matured, usually contain oxygen generated by photosynthesis, making them float and drift with the current, reaching long distances (Song *et al*. 2019), and they buoyed up to the surface of the water and float away (Blackler 1967). Some *Colpomenia* species have a tendency to appear in greenish color where it varies within populations and relates to time length immersed and temperature, or due to any inherent characteristics (Clayton 1975). Since the establishment of the genus *Colpomenia* and earlier studies of this genus, there are several key morphological groups that were categorized: species having hollow-globular, species that are elongated or tubular and species that have branching thallus. Such morphological differences listed above, Cho *et al*. (2006) mentioned that there’s a need for revision of the genus.

The genus *Colpomenia* includes nine taxonomically accepted species (Guiry & Guiry 2022): *C*. *claytoniae* S. M. Boo, K. M. Lee, G. Y. Cho *et* W. Nelson (Boo *et al*. 2011), *C*. *ecuticulata* M. J. Parsons (Parsons 1982), *C*. *expansa* (D. A. Saunders) Y.-P. Lee (Lee 2008), *C. hasanainii* Aisha & M. Shameel (*nom*. *inval*.) (Aisha & Shameel 2012), *C*. *mollis* W. R. Taylor (Taylor 1945), *C*. *nainativensis* Durairatnam (Durairatnam 1962), *C*. *peregrina* Sauvageau (Sauvageau 1927), *C*. *ramosa* W. R. Taylor (Taylor 1945) and, *C*. *sinuosa* (Mertens *ex* Roth) Derbès *et* Solier (Castagne 1851). With an addition of new species, *C*. *borea* (Dy, M. Hoshino, T. Abe, Yotsukura, K. M. Lee, S. M. Boo, N. Klochkova & Kogame 2022), making a total of ten currently recognized species. To further discuss this genus *Colpomenia*, I briefly listed and provided some descriptions and

photomicrographs of some representative species collected in Japan (Fig. 1 a, b, c, d) that constitute this genus below.

***Colpomenia claytoniae* S.M.Boo, K.M.Lee, G.Y.Cho & W.Nelson 2011** (Fig. 1c)

Boo *et al*. 2011 160, figs 1a–f; Holotype (SMB000001, in CNUK, Herbarium of Chungnam National University, Daejeon, Korea; 12 January 2005), Isotypes (SMB000002–7), Paratypes (SMB000009–20); Type locality: Sangjokam, Goseong, Korea; Up to 30 cm diameter, yellowish-green to brownish color, globose or vesicle-like, irregularly convoluted thalli, pigmented membrane thickness of up to 300 µm, up to two layered polygonal cortex; up to 6 cuboidal medullary cells, single-celled plurilocular sporangia, uni- and biseriate with up to 8 locules, single-celled same height as plurilocular sporangia, hairs arising from cortical cells; Epilithic on rocks in the lower intertidal zone; Distribution: California, South Africa, Hong Kong, Japan, Korea, Australia and New Zealand.

***Colpomenia ecuticulata* M.J.Parsons 1982**

M.J.Parsons 1982, 297, figs 8–10, 15; Holotype (Hawkes, CHR 357286; 14 January 1980); Type locality: Takatu (Tawharanui) Peninsula, south side of Omaha Bay, near Warkworth, east coast, North Island, New Zealand; 30 cm or more length, 2.5 cm diameter, yellowish-brown in color, slightly folded, globose or vesicle like thalli, up to two layered angular cortex, up to 4 layered cuboidal medullary cells, biseriate with up to 6 locules, 3 celled paraphyses longer than plurilocular sporangia, scattered hair pits arising from medullary cells; Epiphytic on *Carpophyllum flexuosum* (Esper) Greville in 3–5 m of water; Distribution: Pakistan, Korea, Australia and New Zealand.

Colpomenia expansa (D.A.Saunders) Y.-P.Lee 2008

Basionym: *Colpomenia sinuosa* f. *expansa* D.A.Saunders 1898; Lee, Y.P. 2008; 116, figs a–d; Holotype (D.A. Saunders, 1898, p. 164, lám. XXXII: figs. 4–6); Type locality: Santa Catalina Island, California, U.S.A.; Up to 6 cm diameter, brown color, globose or vesicle-like, very tiny clamps of hair on the surface, up to 3 layered angular cortex, up to 7 layered cuboidal medullary cells, single celled same height as plurilocular sporangia, multiseriate up to 8 locules, hairs arising from cortical cells; Epilithic on rock in intertidal; Distribution: Mexico and Korea.

Colpomenia hasanainii Aisha & M.Shameel 2012 (nom. inval.)

Aisha & M.Shameel 2012; 124, fig. 2 a–d; Holotype: specimen place, the herbarium or collection or the institution where the type was stored was not specified; Type locality: Buleji, Karachi, Pakistan; Up to 7 cm diameter, light brown color, irregularly lobed (half part convoluted and half with tuberculae), up to 4 layered squared cortex, cortical region consisted of two: outer having small single layered angular cells and inner having large cells with no definite shape; cylindrical plurilocular sporangia, usually uniseriate, cuticle absent, no unilocular sporangia, oblong paraphyses, hairs absent; Epilithic on rocks in low tides; Distribution: Pakistan.

Colpomenia mollis W.R.Taylor 1945

Taylor, W.R. 1945; 12: i-iv, 1–528, 3 figs, 100 pls.; Holotype (W.R. Taylor, 34-491A; 12 February 1934); Type locality: Isla Gorgona, Valle, Colombia; Up to 25 cm tall, 2.5 cm in diameter, golden to dark brown in color, compressed sacs with short branches and spine-like protuberance thalli, one layered sub-quadrangular

cortex, single layer angular medullary cells, no phaeophycean hair has been studied; Scarcely epilithic on rocks in littoral pools; Distribution: Colombia.

Colpomenia nainativensis **Durairatnam 1962**

Durairatnam 1962; 6–7, pl. 1; Holotype (W.R. Taylor, 34-491A; 12 February 1934); Type locality: Nainativu Island, Sri Lanka; Up to 6 cm tall irregularly lobed hollow, short, rounded branches truncate at ends bearing spine-like projections thalli, a single layered angular cortex, about 3 layered medullary cells, no phaeophycean hair has been studied; Growing on rocks in littoral zone; Distribution: Sri Lanka.

Colpomenia peregrina **Sauvageau 1927** (Fig. 1b)

Sauvageau 1927: 321, figs 1-8; Holotype (Herb Sauvageau, PC (Womersley 1967, p. 244)); Lectotype (Brittany, France, PC; herb); Type locality: Morbihan, France; Up to 10 cm tall, greenish brown, smooth, globose, up to 300 μm thick, up to 5 small polygonal cortical layers, medulla up to 4 layers, extensive plurilocular sporangia, one-celled long paraphysis; Usually epiphytic, rarely epilithic in intertidal to subtidal; Distribution: Azores, Canary Islands, Madeira, Lanzarote, Savage Islands, Baltic Sea, Britain, Bulgaria, Channel Islands, Denmark, France, Greece, Ireland, Isla de Alborán, Italy, Netherlands, Norway, Portugal, Scandinavia, Spain, Israel, Turkey, Alaska, Aleutian Islands, British Columbia, California, Gulf of California, Maine, Massachusetts, Mexico, New Hampshire, Nova Scotia, Oregon, Rhode Island, Washington, Algeria, Liberia, Morocco, Spanish North Africa, Tanzania, Lebanon, Pakistan, Japan, Korea, Russia, Russia Far East, Australia, New Zealand, Hawaiian Islands, and Solomon Islands.

***Colpomenia ramosa* W.R.Taylor 1945**

W.R.Taylor 1945: 84, pl. 6: fig. 2; Type (W.R. Taylor no. 34-651 (AHFH); 10 March 1934); Type locality: South Bay, Isla Cerros, Baja California, Mexico; Up to 2 cm tall and 4 cm wide forming adherent clumps forming multiple attachments, crisp to stiff, irregular subdichotomously to polychotomously branched, small celled cortical cells, up to 8 medullary cells layers, uniseriate up to 12 locules, no recorded paraphysis and phaeophycean hairs; Growing on littoral pools; Distribution: Baja California, Gulf of California, Mexico, Costa Rica, and Galápagos Islands.

***Colpomenia sinuosa* (Mertens ex Roth) Derbès & Solier 1851** (Fig. 1a)

(Mertens ex Roth) Derbès & Solier 1851: 95; Type: "Inter Algas e mari Atlantico prope Gades" [near Cádiz, Spain]; (Roth 1806: 327); Type locality: Mertens; BM; Type material: B, destroyed, unconfirmed herbarium desposition of other type material; Up to 15 cm diameter, broad, firm, deeply folded globose or vesicle-like thalli, up to 6 layers pigmented polygonal cortex, more or less than 500 μm thick, up to 6 medullary layers, single-celled paraphysis, punctate associate with a hair pit, epilithic on rocks and occasionally epiphytic in lower intertidal to subtidal; Distribution: Canary Islands, Cape Verde Islands, Gough Island, St Helena, Adriatic Sea, Apulia, Channel Islands, France, Greece, Italy, Netherlands, Portugal, Spain, Alaska, Baja California, British Columbia, California, Florida, Georgia, Gulf of California, Islas Revillagigedo, Mexico, North Carolina, Washington, Belize, Costa Rica, Panama, Veracruz (State), Bahamas, Barbados, Caribbean, Cuba, Jamaica, Martinique, Netherlands Antilles, Puerto Rico, Trinidad & Tobago, Virgin Islands, Tropical and Subtropical West Atlantic, Argentina, Brazil, Chile,

Colombia, Galápagos Islands, Peru, Uruguay, Venezuela, Algeria, Angola, Côte d'Ivoire, Equatorial Guinea, Eritrea, Ethiopia, Gabon, Ghana, Guinea-Bissau, Kenya, Libya, Madagascar, Mauritania, Morocco, Mozambique, Namibia, São Tomé & Príncipe, Senegal, Sierra Leone, Somalia, Andaman Islands, Laccadive Islands, Mauritius, Réunion, Seychelles, Arabian Gulf, Bahrain, Cyprus, Egypt, Iran, Israel, Jordan, Kuwait, Levant Basin, Oman, Red Sea, Saudi Arabia, Syria, Turkey, Yemen, Abu Dhabi, Bangladesh, India, Pakistan, Sri Lanka, West Bengal, Indonesia, Malaysia, Myanmar (Burma), Philippines, Singapore, Thailand, Vietnam, China, Hong Kong, Japan, Korea, Kuril Islands, Russia Far East, Sakhalin Island, South China Sea, Taiwan, Yonaguni Island, Australia, New Zealand, Central Polynesia, Easter Island, Federated States of Micronesia, French Polynesia, Guam, Hawaiian Islands, Mariana Islands, New Caledonia, and Îles Kerguelen; Remarks: According to Silva *et al*. (1996), the names *Asperococcus sinuosus* var. *lobatus* and *Stictosiphon cavernosus* var. *lobatus* are invalid referable to this species. Although it has not yet been transferred or reduced to synonymy, the taxon *Asperococcus sinuosus* var. *firmior* Sonder appears to fall within the circumspection of this species (Silva *et al*. 1996: 629). According to Papenfuss (1964: 19), the Antarctic records might be based on *Caepidium antarcticum*'s vesicular stage.

As mentioned earlier, the use of multiple molecular markers has been proven effective for phylogenetic studies. This study has benefited from those advantages, and it is why I opted to use them here. First is the use of cytochrome oxidase subunit 3 or *cox*3. It is a mitochondrial gene that can be utilized as a molecular marker for phylogenetic study because it is a fast-evolving gene. This gene can be used to infer relationships between groups of species that are

distantly related, proven for unraveling hidden diversity, and revealing distribution patterns (Kogame *et al*. 2005; Uwai *et al*. 2006; Boo *et al*. 2011). In addition, it is useful and effective for species identification, phyletic evolution, and genetic structure (Galtier *et al*. 2009; Cai *et al*. 2017; Chen *et al*. 2019). Second, using the Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RuBisCo) or *rbc*L gene. This is located in the large single-copy gene stage in chloroplast genome which means it adapted early before the event of gene duplication, that have already adapted (Strauss *et al*. 1988; Bausher *et al*. 2006; Piatigorsky 2007; Sen *et al*. 2011). This gene is used widely for DNA markers across all species of algae, such as: the low evolutionary rate, simplicity to amplify and sequence all variety of photosynthetic taxa, and this gene is the most abundant protein in nature (Sen *et al*. 2011). And third is using the photosystem I P700 chlorophyll a apoprotein A1 or simply *psa*A that encodes for the photosystem I (PSI) complex, which is crucial for the primary light reaction in photosynthesis. The *psa*A gene is conserved and has slow evolutionary rate, similar to the *rbc*L gene, because this system has remained relatively consistent throughout evolution where it is less prone to several mutations that might have recently taken place.

The mitochondrial *cox*3 has been used in a lot of studies for species identification and investigating genetic variations (interspecific and intraspecific levels) in brown algae. My *rbc*L and *psa*A sequence data confirmed a single evolutionary origin for the family Scytosiphonaceae and were consistent with clear resolutions, similar to those published earlier (Kogame *et al*. 1999; Cho *et al*. 2001, 2003, 2006).

AIMS AND OUTLINES OF THIS THESIS

However, despite continuous revisions and studies within the phylogenetic relationships of the Scytosiphonaceae using multiple gene sequences, such as nuclear SSU rDNA, LSU rDNA and ITS, and plastid *rbc*L, RuBisCo spacer and *psa*A, polyphyly and paraphyly has been present among scytosiphonaceaen genera (Kogame *et al*. 1999, 2011; Cho *et al*. 2006; Kain (Jones) *et al*. 2010; Boo *et al*. 2011; Lee *et al*. 2014b).

In Chapter 2, a new species of the genus *Colpomenia* has been described from Hokkaido, Japan and in Magadan, Russia using *cox*3 and *rbc*L molecular tools together with morphological observations and life-history study.

In Chapter 3, the combination of these genes (*cox*3, *rbc*L and *psa*A) will provide more insight into the classification of the family Scytosiphonaceae and allow more detailed understanding. This also focuses on resolving conflicts within the *Colpomenia* species by suggesting several proposals to address issues in this genus.

And in Chapter 4, species delimitation and geographical distribution based on *cox*3 sequence data as an input using intraspecific and interspecific divergence for ABGD and ASAP, and non-ultrametric phylogenies as an input to use the number of substitutions for PTP and bPTP. These species delimitation analyses allow us to take a closer look at several groupings or partitioning of each putative species.

Chapter 2.

Colpomenia borea a new species of *Colpomenia* from Hokkaido, Japan and Far East Russia

INTRODUCTION

The brown algal family Scytosiphonaceae (Ectocarpales) is distinguished from other families by having one cup-shaped chloroplast with a single pyrenoid per cell. This family has a heteromorphic life history in which an erect gametophytic thallus alternates with a prostrate sporophytic thallus, except for *Melanosiphon* and *Myelophycus*, which have an isomorphic life history (Wynne 1969; Nakamura & Tatewaki 1975; Kawai *et al*. 1994; Cho *et al*. 2003).

Colpomenia (Endlicher) Derbès *et* Solier is a scytosiphonacean genus and is widely prevalent in temperate to tropical waters around the world (Boo *et al*. 2011). This genus has been relatively well studied in its molecular phylogeny using cytochrome oxidase subunit 3 (*cox*3) and RuBisCo large subunit (*rbc*L) genes (Cho *et al*. 2005; Boo *et al*. 2011; Lee *et al*. 2012, 2013, 2014a). The

Colpomenia traditionally included various forms of hollow thalli which were globular, tubular, and branched (Wynne & Norris 1976). However, three tubular *Colpomenia* species were recently transferred to the genus *Dactylosiphon* [*D. bullosus* (D. A. Saunders) Santiañez *et al*., *D. durvillei* (Bory) Santiañez *et al*., and *D. wynnei* (K. M. Lee *et al*.) Santiañez *et al*.] based on their molecular phylogeny and morphology of gametophytic and sporophytic thalli (Santiañez *et al*. 2018). In addition, *Colpomenia tuberculata* D. A. Saunders which has thick and convoluted hemispherical thalli with tubercles was recently transferred to the genus *Encephalophycus* [*E. tuberculatus* (D. A. Saunders) Santiañez] (Santiañez 2022).

Although the *Colpomenia* species have been relatively well studied, there was no taxonomic study using molecular analyses in *Colpomenia* from Hokkaido, Japan and the Russian Far East. In this study, I investigated *Colpomenia* species from the regions using morphological and molecular analyses. Based on the results, I proposed a new species of *Colpomenia* as *Colpomenia borea* sp. nov. (Dy *et al*. 2023).

MATERIALS AND METHODS

Collection of *Colpomenia* samples

Colpomenia samples were newly collected in this study from Akkeshi, Hokkaido, Japan (43°01'16.9"N, 144°50'12.5"E) intermittently in 2011–2017 and from Magadan, Russian Far East (59°29'30.5"N, 150°42'17.4"E) on 11 and 15

July 2016 (Figs. 2, 3, Table 5) in an intertidal zone and a subtidal zone by a long hand net or scuba diving. From these samples, specimens were pressed as voucher herbarium specimens, and parts of them were dried in silica gel for molecular analyses. Voucher specimens were deposited in the Herbarium of the Faculty of Science Hokkaido University (SAP), Sapporo, Hokkaido, Japan, and Kamchatka State Technical University (KamchatGTU), Petropavlovsk-Kamchatsky, Russia. I additionally examined nine specimens of *Colpomenia* found in SAP. (Tables 4, 5).

Morphological Analysis

For morphological analysis, sections of thalli were made by hand using a razor blade and were stained with cotton blue in a lactic acid-phenol-glycerol water (1:1:1:1) solution and mounted on microscope slides. Photomicrographs were taken using a Nikon Eclipse E600 (Nikon, Tokyo, Japan) with a Nikon Digital Sight DS-Fi1-L2 (Nikon, Tokyo, Japan).

Culture Isolates

Unialgal isolates from Akkeshi samples, collected on 9 July 2017 were established by plurispores (putative gametes) released from a globose thallus using a 48-well plate. Each germling of putative gametes was transferred into an individual well in the plate containing PESI medium (Tatewaki 1966), and GeO_2 (0.005 mg ml^{-1}) (Kogame & Yamagishi 1997) was added to prevent the growth of diatoms. Five unialgal isolates from one individual were established in culture.

Cultures were inoculated in plastic Petri dishes (90 × 20mm) with PESI medium under long-day (16:8 h light:dark) and short-day (8:16 h light:dark) conditions with a light density of 30–50 μmol m^{-2} s^{-1} at 10°C, 15°C and 20°C.

Molecular Analyses

Total genomic DNA was extracted using QuickExtract FFPE DNA Extraction Kit (Epicentre, Madison, Wisconsin USA) from silica gel-dried samples following the instructions of the manufacturer. Extracted DNA was then used as a template for PCR to amplify *cox*3 and *rbc*L genes. The primers used in this study were indicated in Table 1 (Yamoto 1997; Kogame *et al*. 1999; Kogame *et al*. 2005; Boo *et al*. 2010).

PCRs were performed using TaKaRa Ex Taq DNA polymerase (TAKARA Bio Inc., Otsu, Japan) added with dimethylsulfoxide (DMSO) under the following conditions: 1 min at 94°C for initial denaturation, followed by 50 cycles of 20 s at 94°C, 20 s at 45°C or 50°C, 45 s at 72°C, followed by a final extension of 7 min at 72°C in GeneAmp PCR System 9600 or 9700 (PE Applied Biosystems, Foster City, California USA). All PCR products were purified using polyethylene glycol (PEG) precipitation and were sequenced using a BigDye Terminator v1.1 or v3.1 Cycle Sequencing Kit (Applied Biosystems, Austin, Texas USA) and an ABI Prism 310 or 3130 Genetic Analyzer (Applied Biosystems).

Together with other related sequences retrieved from GenBank, the newly generated *cox*3 and *rbc*L sequences (Table 5) were aligned using ClustalW in MEGA X (Kumar *et al*. 2018). A total of 48 *cox*3 sequences were successfully aligned, including seven sequences of *Colpomenia borea* from Akkeshi and

Magadan; 15 sequences of other *Colpomenia*, 24 sequences of other scytosiphonacean species, and *Ectocarpus siliculosus* (Dillwyn) Lyngbye, *Pylaiella littoralis* (Linnaeus) Kjellman and *Chordaria flagelliformis* (O.F. Müller) C. Agardh as an outgroup from GenBank. The newly generated *rbc*L sequences were aligned with 37 downloaded sequences of scytosiphonacean species, and *E. siliculosus* and *Ch. Flagelliformis* as an outgroup. Uncorrected p-distances were calculated using MEGA X. Phylogenetic analyses were performed with MEGA X using the Maximum likelihood (ML) method (run on 500 pseudoreplicates in *cox*3 tree and 1000 in *rbc*L tree) and the Tamura-3 parameter model which was selected as the best-fit model by Akaike Information Criterion based on Nearest-Neighbor-Interchange (NNI). Bayesian inference (BI) analyses were performed using MrBayes on XSEDE v3.27a (Ronquist *et al*. 2012) on CIPRES Portal (Miller *et al*. 2010). The BI analyses were conducted with GTR + Γ + I model. Two sets of four Markov chain Monte Carlo chains were run for 1×10^6 generations with sampling of every 100 generations. The first 25% of sampled trees was discarded, and remaining trees were saved for the consensus tree to calculate Bayesian posterior probability values.

RESULTS

Colpomenia borea Dy, M. Hoshino, T. Abe, Yotsukura, K.M. Lee, S.M. Boo, N. Klochkova *et* Kogame, 2023

Description: Thalli, globose to ovoid, smooth, up to 5 cm in diameter; epiphytic; pigmented cortical layer composed of one to two irregularly arranged polygonal

cells of 5.4–14.9 μm in diameter; medulla of two to three cell layers, irregularly shaped colorless cells; paraphyses unicellular and 8–18 μm broad and 7.8–22.5 μm long; plurilocular sporangia (putative gametangia) up to five locules in height and 4.2–5.5 μm × 19.5–30.5 μm in size (Fig. 4).

Holotype: SAP115473 (Fig. 4a), Akkeshi, Hokkaido, Japan (43°01'16.9"N, 144°50'12.5"E), 10 July 2013; deposited in SAP.

Etymology: From the Latin 'boreus', meaning 'northern'.

Specimens examined: Specimens examined in this study are listed in Table S1.

Ecology: Epiphytic on a sargassacean alga, *Stephanocystis crassipes* (Mertens ex Turner) Draisma *et al*. (Fig. 4a, arrows). Growing in the intertidal to subtidal of wave-protected areas in Akkeshi, Japan (Fig. 3) and Magadan, Russia.

Distribution: This species was found in the Pacific Coast of Hokkaido, Japan and Magadan, Russian Far East.

Morphological observation

Thalli were up to 5 cm in diameter, globose to ovoid, hollow, flaccid, golden-brown in color (Fig. 4a, b), becoming pale brown when dried on herbarium sheets, epiphytic on a sargassacean alga, *Stephanocystis crassipes* (Fig. 4a, arrows).

In surface view, surface cells were polygonal 6.3–13.2 μm in diameter and arranged irregularly, and ascocysts (paraphyses) and plurilocular sporangia (putative gametangia) were observed (Fig. 4c). In cross section, thalli were very thin (150–210 μm) with a cortex of one to two pigmented polygonal cells (6.9–20

μm in diameter) (Fig. 4d). Putative gametangia were formed with ascocysts (paraphyses) on an entire surface and up to five locules in height (Fig. 4e). Putative gametangia were uniseriate and 4.2–5.5 μm broad and 19.5–30.5 μm long. Ascocysts were ovoid and 8–18 μm × 7.8–22.5 μm in size. And a medullary layer, irregularly shaped colorless cells (up to 125 μm in diameter) with two to three layers (Fig. 4f). Phaeophycean hairs were immersed in pit and arising from the cortical cells (Fig. 4g). Putative gametangia were formed with ascocysts (paraphyses) on an entire surface and up to five locules in height (Fig. 4e). Putative gametangia were uniseriate and 4.2–5.5 μm broad and 19.5–30.5 μm long. Ascocysts were ovoid and 8–18 μm × 7.8–22.5 μm in size.

Culture

Plurispores (putative gametes) released from a globose thallus were cultured at 15°C in a long-day condition. The putative gametes germinated unipolarity (Fig. 5a) without gamete fusion and developed into pseudodiscoid thalli about 5 mm in diameter (Fig. 5b, c), which formed broadly ellipsoid to rectangular shape of plurilocular sporangia (16–20.8 μm × 24–40 μm in size) and ascocysts (9.8–22.4 μm × 17.3–28.1 μm in size) (Fig. 5d).

The plurispores released from the pseudodiscoid thalli were cultured at 10°C, 15°C and 20°C under long-day and short-day conditions. Settled plurispores were 3.5–8.1 μm in diameter, and they developed into pseudodiscoid thalli which formed plurilocular sporangia under a long-day condition and unilocular sporangia at 10°C under a short-day condition (Fig. 5e). Unilocular

sporangia were ellipsoid and 12.3–35.8 μm × 14.9–40.9 μm in size. Unispores from unilocular sporangia developed into small saccate thalli with phaeophycean hairs (Fig. 5f, Fig. 4). *Colpomenia borea* life history diagram in culture has been drawn in Fig. 6.

Phylogeny of *cox*3 and *rbc*L

The length of the *cox*3 alignment was 746 bases. The ML and BI trees showed highly congruent tree topologies. In the ML tree of *cox*3 (Fig. 7), *C*. *borea* (two samples from Magadan and four samples from Akkeshi) formed a highly supported clade (with 98% in BP and full support in PP). The clade of *C. borea* was sister to the clade of *C*. *claytoniae*, *C*. *expansa*, and *C*. *peregrina*. Intraspecific sequence differences (uncorrected p-distances) of *C*. *borea* were less than 0.17%, while sequence differences between *C*. *borea* and other *Colpomenia* were more than 8.5%.

Two samples of *C. borea* collected from Akkeshi, Hokkaido and Magadan, Russia were sequenced for *rbc*L. The length of the alignment was 1467 bases. In my phylogenetic analyses (Fig. 8), the two sequences of *C*. *borea* formed a distinct clade (BP = 98%, PP = 1.0), and the clade of *C*. *borea* clustered with *C*. *expansa C*. *claytoniae* and *C*. *peregrina*.

Species of *Colpomenia* did not form a clade in both *cox*3 and *rbc*L trees. But *C*. *claytoniae*, *C*. *expansa*, *C*. *peregrina*, and *C*. *borea* showed to be monophyletic in both trees (BP = 97% and PP = 1.0 in *cox*3, BP = 99%; PP = 1.0 in *rbc*L).

DISCUSSION

The genus *Colpomenia* shows various thallus forms: i.e., globular thallus with smooth surface (*C*. *claytoniae*, *C*. *ecuticulata*, *C*. *expansa*, and *C*. *peregrina*), hemispherical thallus with tubercles (*C*. *hasanainii*) and branched thallus (*C*. *mollis*, *C*. *nainativensis* and *C*. *ramosa*) (Kogame 2001; Kogame & Masuda 2001; Cho *et al*. 2006; Aisha & Shameel 2012; Lee *et al*. 2014b; McDevit & Saunders 2017; Santiañez *et al*. 2018; Santiañez 2022). *Colpomenia sinuosa* usually have globular thalli with smooth surface but sometimes shows a tuberculate surface (Lee *et al*. 2013). Since *C*. *borea* has globular thalli with smooth surface, the species should be compared with the five *Colpomenia* showing the similar thallus form: i.e., *C*. *claytoniae*, *C*. *ecuticulata*, *C*. *expansa*, *C*. *peregrina* and *C*. *sinuosa*. In my *cox*3 analyses, all the five species were included. The *cox*3 analysis indicated that *C*. *borea* is clearly separated from seven *Colpomenia* species including the five globose species. *Colpomenia borea* was relatively closely related to three globose species, *C*. *peregrina*, *C*. *expansa* and *C*. *claytoniae* in the *cox*3 analyses. But the *cox*3 sequences of *C*. *borea* were different from those of the three species by 8.5–10%, while sequence differences within *C. borea* were less than 0.17 %. These results indicate that *C*. *borea* is an independent species, suggesting that it is a new species.

Also, in my *rbc*L analyses, it was supported that *C*. *borea* is clearly separated from *C*. *claytoniae*, *C*. *expansa* and *C*. *peregrina*. However, the separation of *C*. *claytoniae* and *C*. *expansa* was unclear. This unclear separation is probably responsible to the low evolutionary rate of the *rbc*L gene which cannot efficiently separate closely related species in the Scytosiphonaceae (Lee *et al*. 2014b; Hoshino *et al*. 2021).

Globular species of *Colpomenia* can be morphologically characterized by the combination of the following thallus characters: shape, size, and thickness of thallus and unicellular/multicellular ascocyst (paraphysis) (Table 2). *Colpomenia borea* is morphologically distinguished from other globular *Colpomenia* by smooth surfaces in possessing the thinnest thalli (150–210 μm). *Colpomenia ecuticulata* also has thin thalli but has multicellular ascocysts (paraphyses) associated with plurilocular sporangia (putative gametangia) in globular gametophytic thalli (Parsons 1982), while *C*. *borea* has unicellular ascocysts. *C*. *borea* has the characteristic of being epiphytic on *Stephanocystis* in areas protected from waves. In all of my *C*. *borea* specimens, *C*. *borea* thalli were epiphytic on *Stephanocystis*. *Stephanocystis crassipes* is distributed in Hokkaido, Sakhalin, Kuril Islands and Magadan (Yoshida 1998; Klochkova *et al*. 2013). It should be noted that *C*. *borea* is distributed in the coldest region in a known distributional range of *Colpomenia* (Wynne & Norris 1976; Lee *et al*. 2013, 2014a). Akkeshi, the type locality of *C*. *borea*, is affected by the cold Oyashio Current. The monthly-average seawater temperatures ranged between 0˚C and 17.5˚C in the last 10 years at Akkeshi (from the website of Akkeshi Marine Station, searched on 1 September 2021).

The majority of *Colpomenia* species are distributed in tropical or warm waters (Wynne & Norris 1976; Lee *et al*. 2013). In the northwest of the Pacific, *C*. *claytoniae*, *C*. *expansa*, *C*. *peregrina* and *C*. *sinuosa* have been reported in China, Korea and/or Japan (Boo *et al*. 2011; Song *et al*. 2019). But, in Hokkaido, northern Japan only *C*. *peregrina* of these species are found (data not shown), excluding *C*. *borea*.

I checked previously published names referrable to *C*. *borea*, other than taxonomically accepted names of *Colpomenia* species. There are some taxonomic synonyms of *C*. *sinuosa*: *Tremella cerina* Clemente y Rubio (Clemente y Rubio 1807), *T*. *rugosula* Clemente (Clemente y Rubio 1807), *Stilophora vesicata* Harvey (Harvey 1834) and *Soranthera leathesiformis* P. Crouan *et* H. Crouan (Schramm & Mazé 1865) (Guiry & Guiry 2022b). Although their original descriptions are brief and insufficient, their thalli are sinuous, rugose, imbricate or lobed. Such thalli are more similar to those of *C*. *sinuosa* than *C*. *borea*. *Tremella cerina* and *T*. *rugosula* were described from Andalucia, Spain. Type localities of *St*. *vesicata* and *Sor*. *leathesiformis* are tropical to subtropical regions: Mauritius and Guadeloupe, respectively. Since these localities are environmentally different from Akkeshi and Magadan where *C*. *borea* grows, it is unlikely that the entities connected to those names are conspecific to *C*. *borea*. *Colpomenia sinuosa* included some forms, but they were raised to a specific rank, except for *C*. *sinuosa* f. *lacunosa* W.R.Taylor (Taylor 1945), which has short projections on surface. Consequently, we concluded that *C*. *borea* from Akkeshi and Magadan is a well distinct new name from previous synonyms.

My cultures of *C*. *borea* showed a heteromorphic life cycle between globose thalli and pseudodiscoid thalli. The pseudodiscoid thalli formed plurilocular sporangia in long-day conditions and unilocular sporangia in short-day conditions. Similar life cycles have been reported in *C*. *peregrina* and *C*. *sinuosa* (Clayton 1979; Kogame 1997; Kogame & Yamagishi 1997; Toste *et al*. 2003). Although sexual reproduction was unknown in *C*. *borea*, it has been reported in *C*. *peregrina* and *C*. *sinuosa* in which globose thalli were gametophytes and pseudodiscoid thalli were sporophytes (Kogame & Yamagishi

1997; Toste *et al*. 2003). Pseudodiscoid thalli of *C*. *borea* are similar to those of *C*. *peregrina* and *C*. *sinuosa* in forming ascocysts in addition to plurilocular and unilocular sporangia. But the shape of plurilocular sporangia on pseudodiscoid thalli of *C*. *borea* was different from those in *C*. *peregrina* and *C*. *sinuosa*. Plurilocular sporangia on pseudodiscoid thalli of *C*. *borea* were not ectocarpoid but broadly ellipsoid to rectangular, while those of the two latter species are ectocarpoid (Kogame 1997; Kogame & Yamagishi 1997; Toste *et al*. 2003). This difference in morphology may also be useful as a taxonomic character to distinguish *C*. *borea* from other *Colpomenia*.

Colpomenia borea probably has been confused with other *Colpomenia* species, especially *C*. *peregrina*. For example, in my survey of the herbarium specimens deposited in SAP, I have found several specimens of *C*. *borea* that have been previously misidentified as *C*. *peregrina* or *C*. *sinuosa*. As such, I expect that further studies on *Colpomenia* specimens collected from colder waters would expand the distributional range of *C*. *borea*.

CONCLUSION

Problems of paraphyly or polyphyly in some scytosiphonacean genera including *Colpomenia* have been shown in previous molecular phylogenetic studies using not only a single gene but also multiple genes (Santiañez *et al*. 2018, 2020). Similarly, members of the genus *Colpomenia* did not form a clade in my analysis; the clade of *C*. *peregrina*, *C*. *claytoniae*, *C*. *expansa*, and *C*. *borea* was

well supported but separated from other *Colpomenia* species including the generitype *C*. *sinuosa*. This may imply that the well supported clade of the four species is a distinct genus from *Colpomenia*. However, further molecular studies using more genes and more taxa, particularly in the '*Hydroclathrus* group' (Santiañez *et al*. 2018), and in some cases, further studies on their life history traits [i.e., life cycle (e.g., morphology of sporophytes) and the sexual system (e.g., iso- or anisogamy)] are required to clearly identify diagnostic characters that will help solve the problems in the molecular phylogeny, taxonomy, and classification of the Scytosiphonaceae.

Chapter 3.

Monophyly of the genus *Colpomenia*

INTRODUCTION

Molecular phylogenetic studies in the family Scytosiphonaceae have pointed out polyphyly or possibility of polyphyly of some genera (Kogame *et al*. 1999; Cho *et al*. 2001, 2006; Santiañez & Kogame 2022). The genus *Colpomenia* was one of such genera. *Colpomenia* included tubular species: *C*. *bullosa*, *C*. *durvillei*, and *C*. *wynnei*. But these tubular species positioned away from the generitype of *Colpomenia* (*C*. *sinuosa*) in phylogenetic trees (Kogame *et al*. 1999; Boo *et al*. 2011; Lee *et al*. 2012). Santiañez (2018) excluded tubular species from *Colpomenia* and proposed the new genus *Dactylosiphon* for the tubular species. Santiañez (2022) also segregated *Colpomenia tuberculata* D.A.Saunders from the genus *Colpomenia* and transferred it to his new genus *Encephalophycus*. However, *Colpomenia* is still possibly polyphyletic even in recent phylogenetic studies (Kogame *et al*. 1999; Cho *et al*.

2001, 2006; Santiañez *et al*. 2018, 2020; Santiañez & Kogame 2022; Dy *et al*. 2023). In this study, phylogenetic analyses were carried out to examine polyphyly of *Colpomenia*, including more taxa and using *rbc*L and *psa*A data.

Colpomenia sinuosa, *C*. *claytoniae*, and *C*. *peregrina* have a wide distribution, and their relatively large infraspecific variations were reported in *cox*3 sequences. In this study, phylogenetic trees of *cox*3 were constructed to re-examine infraspecific variations of *Colpomenia*, including downloaded data from GenBank and samples collected from Japan.

Four species of globular *Colpomenia* have been reported in Japan: *C*. *sinuosa*, *C*. *claytoniae*, *C*. *peregrina*, and *C*. *borea*. These species are similar to each other in gross morphology and have been misidentified. Thus, Japanese *Colpomenia* is needed to be reexamined using molecular analyses. In this study, *Colpomenia* samples collected from various localities of Japan were used for *cox*3 analyses and were molecularly identified.

MATERIALS AND METHODS

Collections of samples

Samples (Tables 4, 5, 6) used in this study were collected from several localities (Fig. 9) from an intertidal zone to a subtidal zone along the coast by a long hand net, floating or drifting, epilithic on a rock or epiphytic on some *Sargassum* species. Portion of thalli from collected specimens were dried on silica-gel and were used

in extraction of DNA, and the remainder of each specimen were pressed on herbarium sheets and were air-dried as voucher specimen. Voucher specimens were stored in SAP.

DNA extraction

Total DNA was extracted in the same method as that of Chapter 2, using GenCheck DNA Extraction Reagent (FASMAC, Atsugi, Japan).

DNA amplification and sequencing

Extracted DNA was used as template for PCR to amplify *cox*3, *rbc*L, and *psa*A genes. Primers used for PCR and sequencing are shown in Table 1. PCR methods were done using TaKaRa Ex Taq DNA polymerase (TAKARA Bio Inc., Otsu, Japan) and/or Q5 High-Fidelity DNA Polymerase (New England Biolabs Inc., Massachusetts, USA) and were run in a thermal cycles either in GeneAmp PCR System 2720 or 9700 (PE Applied Biosystems, Foster City, California USA) and/or GeneAtlas G G02 (Astec Bio, Fukouka, Japan) under these conditions: for the *cox*3 and *rbc*L genes, 1 min at 94°C for initial denaturation, followed by 45 or 50 cycles of 20 s at 94°C, 20 s at 45°C or 50°C, 45 s at 72°C, followed by a final extension of 7 min at 72°C; and for *psa*A genes, initial denaturation for 5 min at 94°C, followed by 37 cycles or 50 cycles of 1 or 5 min at 94°C, 1 min at 45°C, 1 min at 72°C,

followed by a final extension of 5 min at 72°C. The quality and quantity of the PCR products were checked using agarose gel electrophoresis.

PCR products were cleaned to remove residual primers and dNTPs using polyethylene glycol (PEG #6000, Nakalai Tesque, Kyoto, Japan) and were sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Austin, Texas USA) or SupreDye v1.1 (AdvancedSeq, LLC., Pleasanton, California, USA) and were sequenced in ABI Prism 310 or 3130 Genetic Analyzer (Applied Biosystems).

Phylogenetic analyses

Chromatograms of these sequences were checked thoroughly and assembled alongside referenced sequences from GenBank using MEGA X (Kumar *et al*. 2018) and DNA Baser ver. 5.12 (Heracle Biosoft).

Sequences of these three genes were trimmed and aligned using ClustalW in MEGA X or MEGA 11. To determine the best-fit evolution substitution model, I used ModelTest-NG v.0.1.7 (Darriba *et al*. 2020) on XSEDE (Stamatakis 2014) and was run on CIPRES Portal (Miller *et al*. 2010) under Akaike Information Criterion and Bayesian Information Criterion: TVM for *cox*3, TrN for *rbc*L, and GTR for *psa*A and GTR for *rbc*L and *psa*A concatenated sequences. Although there are multiple different models, I chose the general time-reversible (GTR) because it is the most common and general model for real-world analysis (Stamatakis 2006, Lee *et al*. 2013). I reconstructed trees to compare TVM and TrN models

with GTR model topologies whether they were similar or not, and the results were identical to each other.

Maximum Likelihood (ML) and Bayesian Inference (BI) were performed on CIPRES Portal. For the ML analyses, RaxML-HPC v. 8.2.12 on XSEDE (Stamatakis 2014). Since the selected model is GTR +G+I nucleotide model was executed in RaxML and run on Rapid Bootstrap/Search for best-scoring ML tree and with 1000 bootstrap pseudoreplicates (BP). BI analyses were conducted with MrBayes on XSEDE v3.27a (Ronquist *et al*. 2012). Two independent sets run simultaneously of four Markov chain Monte Carlo chains (one cold chain and three heated chains) were run for 2,000,000 generations with sampling of every 100 generations until the standard deviation of split frequencies fall below 0.01, which indicate convergence iterations. The first 30% of sampled trees was discarded, and remaining trees were saved for the consensus tree to calculate Bayesian posterior probability values (PP). All trees were visualized and constructed using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and Adobe® Illustrator® v27.5.

Additionally, pairwise sequence divergences (p-distances) were calculated either in MEGA X or MEGA 11 (Tamura *et al*. 2021), positions of codons were 1st+2nd+3rd+Noncoding included and all ambiguous positions were deleted for each sequence pair.

Results

Phylogenetic results

Phylogeny of the Scytosiphonaceae based on newly generated sequences from individual samples from each gene (58 *cox*3, 14 *rbc*L, and 17 *psa*A sequences) were inferred together with other downloaded scytosiphonaceaen species from GenBank.

For *cox*3, a total of 203 sequences were aligned (final alignment of 593 bases), including newly generated sequences, other 93 *Colpomenia* sequences, 50 sequences of other scytosiphonacean species, and two outgroup sequences (Fig. 10). A total of 73 sequences were aligned for the *rbc*L (final alignment length of 1466 bases), with newly generated sequences, other 16 sequences of *Colpomenia*, 42 sequences of other scytosiphonacean species, and four outgroup sequences (Fig. 11). For *psa*A, 63 sequences were aligned (final alignment of 1487 bases) with newly generated sequences, five species of other *Colpomenia*, 26 other scytosiphonacean sequences, and three outgroup sequences (Fig. 12). And for concatenated (*rbc*L+ *psa*A), a final alignment of 2953 bases, with newly generated sequences, three species of other *Colpomenia*, 20 other scytosiphonacean sequences, and two outgroup sequences (Fig. 13). All outgroup species were: *Adenocystis utricularis* (Bory) Skottsberg, *Ectocarpus siliculosus* (Dillwyn) Lyngbye, *Chrodaria flagelliformis* (O.F. Müller) C. Agardh, and *Pylaiella littoralis* (Linnaeus) Kjellman.

Bootstrap support (BP) and Posterior Probability (PP) for branches in the trees here were considered as: 'highly supported' if BP ≥ 95 % and PP ≥ 0.98, 'strongly supported' if BP ≥ 70% and PP ≥ 0.95, and 'poorly supported' if BP < 70% and PP < 0.90.

*Cox*3 based phylogeny

In *cox*3 phylogenetic tree (Fig. 10), the genus *Colpomenia* displayed four major clades (Lineages I–IV) and their unresolved relationships within the Scytosiphonaceae.

In *cox*3 pairwise divergence of *C*. *sinuosa* (Lineage I), high sequence variations were observed. In interspecific variations, *C*. *sinuosa* differentiated among its closest globular *Colpomenia* species: *C*. *ecuticulata* (0.122–0.152), *C*. *claytoniae* (0.110–0.141), *C*. *expansa* (0.117–0.133), *C*. *peregrina* (0.108–0.136), and *C*. *borea* (0.119–0.138). On the other hand, *C*. *sinuosa* is differentiated with the other genus of *Hydroclathreae* tribe, *Tronoella* (0.105–0.129), *Hydroclathrus* (0.110–0.150), *Rosenvingea* (0.098–0.133), *Pseudochnoospora* (0.117–0.138), *Encephalophycus* (0.183–0.199), and *Chnoospora* (0.155–0.180).

Intraspecific variations in *C*. *sinuosa* were up to 0.072. Taking a closer look in this gene tree revealed twenty-two sequences of *C*. *sinuosa* clade forming two groups: Group I and II (Fig. 14). Intraspecific variations among group I were up to 12% and group II up to 13%, respectively. In 'Group I', a total of thirteen sequences from France, Korea, Japan, Portugal, South Africa, Spain, and the USA were included. This is strongly supported branch (BP: 86%; PP: 0.92), while subgroups Ia and Ib were poorly to strongly supported. In 'group II', a highly supported branch (BP: 97; PP: 1.0) were consisted of nine sequences from Australia, Japan, Philippines, and Taiwan. Group II subclades IIa and IIb were poorly to strongly supported.

Colpomenia ecuticulata (Lineage III) formed its own clade consisting of only two samples from New Zealand (Fig. 14). It was located beneath its closest

globular sister-taxon, *C*. *sinuosa*. *Colpomenia ecuticulata* and *C*. *sinuosa* differed from each other in a pairwise divergence sequence of 0.122–0.152.

Lineage II was a monophyletic clade composed of *C*. *claytoniae*, *C*. *expansa*, *C*. *peregrina*, and *C*. *borea* (Fig. 15). The branch of this clade was strongly supported (BP: 81%; PP: 1.0). They were differentiated by 0.108–0.176 from *C*. *sinuosa*, 0.118–0.138 from *C*. *ecuticulata* and this monophyletic group differed from the other genera more than 0.190.

The clade of *C*. *borea* consisted of seven samples of Chapter 2 and three additional samples from Japan and was highly supported (BP: 100%; PP: 1.0). Infraspecific divergence of this species was very small up to 0.002. A single subclade can be found composed of two sequences from Magadan, Russia.

The clade of *C*. *claytoniae* was strongly supported (BP: 79%; PP: 1.0) and infraspecific divergence was up to 0.047. This clade branched into two groups, Groups I and II. Poorly supported clade of the 'Group I' (BP: 69%; PP: 0.99) consisted of twenty-nine sequences from Australia, Hong Kong, Japan, Korea, New Zealand, South Africa, and USA with sequence difference of up to 0.029. On the other hand, strongly to highly supported clade (BP: 95%; PP: 0.99) of the 'Group II' (sequence differences of 0.020) consisted of seventeen sequences from Korea, Japan, and USA. The sequence differences between two groups were 0.006–0.033.

C. *expansa* was a highly supported clade (BP: 100%; PP: 1.0) and consisted of two sequences from Korea. This taxon was sister to *C*. *claytoniae*.

The clade of *C*. *peregrina* showed a strong support (BP: 76%; PP: 0.98) with sequence differences of up to 0.040; has several partition groups and each group has several subclades from poorly to strongly supported consisted of sixty-eight

sequences from Japan, Korea, Russia, Australia, New Zealand, Mexico, France, Norway, and Ireland.

Lineage IV was composed of only one species, *C. ramosa* (Fig. 16). It was sister to *Encephalophycus tuberculatus* with a highly support (BP: 99%; PP: 1.0). Higher sequence divergences were observed between *C. ramosa* and *C. sinuosa* (0.148–0.169), 0.150 between *C. ecuticulata*, 0.146 between the monophyletic species group (Lineage II), and the rest of other genera from the tribe is up to 0.165.

_Rbc_L based phylogeny

The *rbc*L phylogenetic tree (Fig. 11) also showed two major clades of *Colpomenia* (Lineages I and II) which corresponded to Lineage I and II in the *cox*3 tree. *Rbc*L sequences of *C. ecuticulata* (Lineage III) and *C. ramosa* (Lineage IV) were not available in the analyses. Lineage I consisted of *Colpomenia sinuosa*, and Lineage II consisted of *C. borea*, *C. claytoniae*, *C. expansa*, and *C. peregrina*. Both Lineages were highly supported (BP: 100%; PP: 1.0). Lineages I and II did not form a clade. But the sister of Lineage I was the clade consisting of *Chnoosopra*, *Iyengaria*, *Encephalophycus*, *Tronoella*, *Rosenvingea*, *Manzaea*, *Pseudochnoospora*, and *Hydroclathrus* with a moderate support (71/1). Lineage II diverged at the basal of the tribe Hydroclathreae. Average sequence divergence between each *Colpomenia* species were 3.1% to 4%. Lineage I of the *rbc*L sequence divergence between *C. sinuosa* and its closest relative species were *C. claytoniae* (0.031–0.040), *C. expansa* (0.033–0.035), *C. peregrina* (0.032–0.038), and *C. borea* (0.035–0.038). Meanwhile, similar, or relatively higher sequence divergences were observed between *C. sinuosa* and several genera in the Hydroclathreae tribe, *Chnoospora* (0.048–0.053), *Iyengaria* (0.042–0.045), *Encephalophycus*, (0.044–

0.048), *Tronoella* (0.034–0.037), *Rosenvingea* (0.041–0.054), *Manzaea* (0.044–0.048), *Pseudochnoospora* (0.039–0.043), and *Hydroclathrus* (0.040–0.044).

Two groups were formed in *Colpomenia sinuosa* (Fig. 17), like in the *cox*3, but less sequences within each group in this phylogeny. A strongly supported clade consisted of eight sequences from Korea and Japan were in 'Group I a and b' including two subgroups; and two samples of highly supported clade (BP: 100%; PP: 1.0) from Australia were in 'Group II'.

In Lineage II (Fig. 18), *C. peregrina* was fully supported, but support values for other *Colpomenia* species were not high. *Colpomenia borea* was strongly supported in BP (BP: 94%; PP: 1.0). *Colpomenia claytoniae* did not make a clade. Subclades within a species were not supported in *rbc*L analyses of these species.

_psa_A based phylogeny

In *psa*A phylogenetic tree (Fig. 12), two highly supported Lineages were also shown with high supports (BP: 100%; PP: 1.0). Lineage I was the clade of *Colpomenia sinuosa*, and Lineage II consisted of *C. borea*, *C. claytoniae*, and *C. peregrina*. Lineages I and II did not make a clade. Lineage I positioned in a clade of other genera in the tribe Hydroclathreae, while Lineage II diverged at the basal node of the Hydroclathreae.

A highly supported (BP: 100%; PP: 1.0) *Colpomenia sinuosa* clade (Lineage I) remained monophyletic and has two groups, 'Group I' had two sequences from Kuwait and 'Group II' was comprised of five samples composed of subgroups II a and b from Korea, Japan, and Australia (Fig. 19).

Lineage II (*C. claytoniae*, *C. expansa*, *C. peregrina*, and *C. borea*) (Fig. 20), the clade of *C. borea* from Japan and Far East Russia was highly supported (BP: 100%;

PP: 1.0). The clade of *C*. *claytoniae* and *C*. *peregrina* was highly supported (BP: 100%; PP: 1.0), but these two species were not clearly separated.

Four sequences of *C*. *claytoniae* from Korea, Japan, Australia, and Portugal were clustered with moderate supports (BP: 94%; PP: 0.9). Meanwhile the *C*. *peregrina* had seven sequences from Korea and Japan and did not form a clade.

Concatenated (*rbc*L + *psa*A) based phylogeny

In the concatenated reconstructed tree (Fig. 13), two major *Colpomenia* clades were also apparent (Lineage I and II) with high supports and did not make a clade, showing polyphyly of *Colpomenia* similar to previous trees. Lineage I was the clade of *C*. *sinuosa* and was highly supported. Lineage I was sister to the clade of *Iyengaria*, *Tronoella*, *Manzaea*, *Rosenvingea*, *Pseudochnoospora*, and *Hydroclathrus*. It was more closely related to *Iyengaria stellata* and *Tronoella ryukyuana* than *Colpomenia* species of Lineage II. Lineage II consisted of *C*. *borea*, *C*. *claytoniae* and *C*. *peregrina*. These species were efficiently segregated with supports.

Discussion

All *Colpomenia* species included here were found within Hydroclathreae tribe proposed by Santiañez (2023). An average sequence divergence for *cox*3 within the genus *Colpomenia* in this study is at least 18% compared to the report by Lee *et al*. (2013); their result was 12%. Such an increased percentage is due to additional samples sequenced.

On the other hand, there was a little saturation observed here in *rbc*L and *psa*A gene regions in this study. However, Cho *et al*. (2004, 2006) reported that their result from these two genes showed no sign of saturation. Probably it could be due to an increased number of sequences. More detailed analyses should be done in the future about the *rbc*L and *psa*A gene saturation.

The Scytosiphonaceae family has been studied in term of their phylogeny and taxonomy but still has possibility of polyphyly in the genus *Colpomenia* (Kogame *et al.* 1999; Cho *et al.* 2001, 2006; Santiañez & Kogame 2022). In this study, *rbc*L and *psa*A sequences of *Colpomenia* were newly generated and were used for phylogenetic analyses to investigate possibility of polyphyly of *Colpomenia*. In the *rbc*L tree, the Lineages I and II of *Colpomenia* were paraphyletic, but both lineages branch support were not high in ML bootstrap analyses. In the *psa*A tree, the Lineage I and II of *Colpomenia* were non-monophyletic with high supports. The *rbc*L and *psa*A concatenated tree also supported polyphyly of the Lineage I and II of *Colpomenia*.

The generitype of *Colpomenia* is *C*. *sinuosa*. Therefore, the four *Colpomenia* species of the Lineage II should be segregated from *Colpomenia*. They are *C*. *borea*, *C*. *peregrina*, *C*. *expansa*, and *C*. *claytoniae*. Any of these species has not been a generitype. Therefore, a new genus name should be proposed for the Lineage II.

The Lineages I and II are morphologically very similar, and it is difficult to distinguish them morphologically at present. But the presence or absence of cuticle covering plurilocular sporangial sori may be a difference between the two Lineages. *Colpomenia sinuosa* has a cuticle on plurilocular sporangial sori, while species of the Lineage II do not have such cuticle (Table 3)(Clayton 1975, 1979;

Kogame 1997; Kogame & Yamagishi 1997; Toste *et al*. 2003; Norris 2010; Lee *et al*. 2013; Song *et al*. 2019).

Colpomenia ecuticulata and *C*. *ramosa* were included in *cox*3 analyses but not included in *rbc*L and *psa*A analyses. In *cox*3 trees, they did not form a clade with either the Lineage I or II. Although their phylogenetic positions have not been revealed in *rbc*L and *psa*A analyses, the two species also may not be included in the genus *Colpomenia*, being distantly related to *C*. *sinuosa*, the generitype of the genus.

Before *Colpomenia ecuticulata* was identified as a different species, it was considered as a young *Hydroclathrus clathratus*, but their characterization is completely different (see Santiañez *et al*. 2018). Although *C*. *ecuticulata* is almost identical to its closely related globular species of *Colpomenia*, they differ from each other in thallus. For example, *C*. *sinuosa*, *C*. *claytoniae*, *C*. *peregrina*, *C*. *expansa*, and *C*. *borea* have a globose or vesicle-like thallus from 5 cm up to 30 cm, while *C*. *ecuticulata* can be characterized by its smooth, usually slightly flattened (sometimes globular to a slightly folded) thallus of up to 50 cm or more and it is covered with blunt projections or pointed tubercles at maturity (Abbot & Hollenberg 1976; Parsons 1982). In cross sections, notable characteristics of *C*. *ecuticulata* amongst other *Colpomenia* species are that it has reticulate sori that are not associated with a hair pit, paraphyses are up to three cells with a subglobose at the upper cell and have thin-celled medullary walls without pits.

These morphological evidence and pattern in *cox*3 trees mentioned above agreed with each other, including those reported by previous researchers. Based on these, *C*. *ecuticulata* should be transferred to a separate genus. However, it also needs another examination and providing more gene sequences.

In *cox*3 tree, *C. ramosa* made a clade with *Encephalophycus tuberculatus* with a high support branch of BP: 99%; PP: 1.0. Since they shared the same clade, there is a common morphological character between them. For example, *C. ramosa* and *E. tuberculatus* possess crisp to coriaceous thalli. However, *C. ramosa* distinctively differs in cylindrical or compressed but very irregular forking interval branches that form adherent clump and has large, broad thalli. Another way to distinguish *C. ramosa* from *E. tuberculatus* is its rounded-end and becoming hollow thalli, size of about 4 cm in diameter and 2 cm tall, has a small-celled cortex, of which one layer is slightly swollen and 6–8 cuboidal medullary cell layers (Table 3). The surface view of *C. ramosa* showed rows of 1–2 pigmented cells arranged ambiguously in rounded-angular longitudinal patterns. In contrast, *E. tuberculatus* exhibits thalli with a diameter of approximately 15 cm, featuring larger cortical or medullary cells. *Colpomenia ramosa* should be also segregated from the genus *Colpomenia*. *Colpomenia ramosa* made a clade with *E. tuberculatus*, but they are different in gross morphology. Therefore, a new genus name may be given to *C. ramosa*.

Confirmed in all phylogenetic trees (Figs. 10, 11, 12), a highly supported clade (BP: ≥ 100%; PP: ≥ 1.0) consists exclusively of *Colpomenia sinuosa* in *cox*3 Lineage I. Fourteen of my samples and sequences from GenBank formed two major groups and under these groups have several subgroups observed within the *C. sinuosa* clade in *cox*3. This species obtained high sequence variations and is congruent to those reported by Lee *et al*. (2013). In the *rbc*L, three newly generated samples and four samples in *psa*A have been added together with other *C. sinuosa* sequences. Larger intraspecific variations in *C. sinuosa* were found also

in *rbc*L and *psa*A analyses than those in other *Colpomenia* species. These results suggest multiple species in *C*. *sinuosa*.

However, Lee *et al*. (2013) indicated that they have not found any morphological differences among this species in their study and all morphological features observed by them correspond to the previous descriptions of *C*. *sinuosa* by these authors: Norris 2010, Womersley 1987, Parsons 1982, Wynne & Norris 1976, and Clayton 1975.

If *C*. *sinuosa* contains more than one species, the problem arises as to which one includes the type. However, the following issues are raised with respect to the type of this species. The description of *Colpomenia sinuosa* is based on Tenerife, Spain by Vandermeulen *et al*. (1984). The authors selected the latter specimen as a neotype specimen keeping the original collection of this species' type locality in Cádiz, Spain collected by Mertens, where this species served as the basis of the genus *Colpomenia*. However, it is believed that the holotype of *C*. *sinuosa* may have been lost or possibly destroyed at Berlin-Dahlem according to Dawson *et al*. (1964). Herbarium search in various locations has been suggested to find *C*. *sinuosa* type collection, hoping for a possibility to find its holotype. One evidence about this species whereabouts was searched by Santiañez in 2018 where he found *C*. *sinuosa* collected from Cádiz on an on-line herbarium called 'Sweden's Virtual Herbarium'. I confirmed that the specimen 'S-A37119', as he mentioned, is currently deposited at the Sweden Museum of Natural History resembled *C*. *sinuosa* illustrated by Roth in 1806 (plate 12b). On this site, I saw an additional information written: '*Mertens scripsit et misit*', which translates to "*Mertens wrote and sent*", which I presume from Mertens. This seemed plausible to assign this as a type specimen rather than the neotype specimen made by Vandermeulen *et al*.

However, I did not find any photo evidence of the resembled *C. sinuosa* specimen on the 'SVH' website. Further investigation regarding the *C. sinuosa* specimen if it is currently deposited in this herbarium. In addition, morphological and molecular characterizations are needed to support the claim.

The Lineage II of the genus *Colpomenia* formed a strongly supported monophyletic cluster in *cox*3 gene. Meanwhile, this cluster is in Lineage II with highly supported clade in *rbc*L and *psa*A phylogenetic trees, including the concatenated tree. This clade was composed of four species, *C. claytoniae*, *C. peregrina*, *C. expansa*, and the newly described species *C. borea*.

Colpomenia borea in Lineage II has been well defined in Chapter 2. Furthermore, the *psa*A gene phylogeny has been documented for the first time in this newly described species, *C. borea*. The latter gene showed *C. borea*'s distinctiveness among other *Colpomenia* species, the same as the two genes inferred earlier. This species in *cox*3 and *psa*A gene trees recovered high bootstrap (BP) and posterior probability (PP) support, except for the *rbc*L tree which has BP: 79%; PP: 1.0. As I observed from all phylogenetic trees made, *C. borea* could probably diverge earlier in the Lineage II.

Three species of *C. claytoniae*, *C. expansa*, and *C. peregrina* were closely related in the Lineage II. *Colpomenia claytoniae* differed from each other by 0–4.7% sequence divergence, a slightly higher than those reported (0–4.55%) by Boo *et al*. (2011) in *cox*3. This species split into two diverse groups, group I and group II. In group I, intragroup distance was up to 0.029, while in group II, intragroup distance was 0.020. This species might include two species. Aside from the *cox*3 groupings, *C. claytoniae* also formed similarly in *rbc*L tree. Based on the above sequences and positions from the tree observed, these two groups have

some differences. Although it is early to make assumptions, there are several bases wherein these two groups may be different from each other. According to Boo *et al*. (2011), *C*. *claytoniae* showed that there are two morphological forms of either large or small thalli for this species; however, they only investigated samples from its type locality in Korea and several paratypes observed from Australia and New Zealand. They have not discussed it thoroughly and there were no samples examined from several other locations where they collected.

In addition, *C*. *claytoniae* also showed a wide distribution around the world and often they were incorrectly interpreted as *C*. *peregrina* variant. Since they were mistakenly referred to as *C*. *peregrina*, their habitat was also in question, where some of the species were either epilithic or epiphytic. Boo *et al*. (2011) suggested a re-examination of specimens in temperate regions worldwide identified as *C*. *peregrina* in algal herbaria.

Colpomenia expansa was well supported in *cox*3 tree, whereas in chloroplast region, it was poorly supported. As repeatedly reported from previous research and results, *C*. *expansa* was sister to the clade of *C*. *claytoniae* and *C*. *peregrina.* The distinguishing feature of *C*. *expansa* from its sister species is the presence of tiny hairs on its thallus surface. Further investigation of *C*. *expansa* is needed and additional sequences in more samples should be analyzed in the future to confirm such preliminary observations.

There are multiple subgroups observed in *Colpomenia peregrina cox*3 tree with sequence divergence of up to 0.040. But clear subgroups were not seen in *cox*3 trees.

Species within the monophyletic clade (Lineage II), *C*. *claytoniae*, *C*. *peregrina*, *C*. *expansa*, and *C*. *borea*, respectively, were all anatomically similar by

having common characters, such as smooth globose to ovoid or vesicle-like thallus. However, during collection of these species in the field, it was sometimes hard to distinguish them. For example, *C*. *claytoniae* was mistaken as a variant of *C*. *peregrina* (Clayton 1979; Cho *et al*. 2005; Boo *et al*. 2011). Another instance of confusion is exemplified by the newly described species, *C*. *borea*, which also has been erroneously associated with *C*. *peregrina*, or has raised uncertainties regarding differentiation between *C*. *peregrina* and *C*. *sinuosa*. But by examining them in great detail, they differed in thallus size, membrane thickness, cortex, cell size, plurilocular sporangia, etc. (see table 3 for comparison) apart from other known species in *Colpomenia* or other genera in the family Scytosiphonaceae. In addition, concatenated phylogeny (Figure 13) has clearly shown and indicated unresolved polyphyly of the genus *Colpomenia*. Additionally, all phylogenetic trees mentioned before also confirmed identical divergence, distinct lineages, and coherent groupings, which makes this monophyletic species clustering an appropriate candidate for establishing a new genus. *C*. *mollis* and *C*. *nainativensis* were also in the genus *Colpomenia*. But, unfortunately these species have no sequence data. Only anatomical observations have been made to distinguish them away from other *Colpomenia* species. However, they share some similarities with each other. These *C*. *mollis* and *C*. *nainativensis* could probably related or sister species or form a separate clade away from the genus *Colpomenia*.

William Randolph Taylor published the description of *C*. *mollis* (Figure 1 f) in 1945 where this species locality can be found in tropical water, scarcely epilithic on rocks in littoral pools. Its type locality is in Isla Gorgona, Valle, Columbia with herbarium number '34–491A (TYPE), 12 Feb. 1934'. This species thallus is small, soft-delicate, compressed, strict-irregularly divided to short or longer than broad

branches with rounded truncate at the ends, some have spinelike projections of up to 25 cm tall and 2.5 cm in diameter, and a single cortical layer, similar to some *Colpomenia* species, and sub-quadrangular cells. *Colpomenia mollis* has a single layered, angular colorless medullary cell. Since this species is delicate and there are very few quantities at hand, Taylor couldn't secure enough sections for cellular measurements and additional morphologies.

Colpomenia nainativensis was described by Durairatnam in Nainativu Island, Sri Lanka growing on rocks in the intertidal zone. This species has an irregular lobed-hollow sac of 5–6 cm tall and 4–7 wide, also has a short irregular of either cylindrical or slightly compressed branch, about 3–5 cm diameter with truncate at the end bearing spinelike projections, similar to *C*. *mollis*. *C*. *nainativensis* cortical composed of a single layer, pigmented angular-shaped cell arranged longitudinally. Its medullary cells were colorless up to three layers. Another similarity that I have observed between these two species above is they have sub-quadrangular shaped cells, but in *C*. *nainativensis* has a smaller outermost layer than two innermost cell layers.

With respect to *C*. *hasanainii*, this is still treated as a part of *Colpomenia* species but has an invalid name. According to Guiry & Guiry (2016), the holotype (illustrated as 'fig. 2d') place was not given nor specified. Naming of a new species or infraspecific taxon should be in accordance with the ICN Art. 40.7 (Rec.40A.3 and 40A.4) rules.

Before I started this study, *C*. *sinuosa*, *C*. *peregrina*, and *C*. *claytoniae* had been reported from Japan. As mentioned above, these species are similar in gross morphology, thus they may have been misidentified. In this study, molecular identification of *Colpomenia* species from Japan were conducted, and their more

accurate distributional ranges were shown (Fig. 9). *Colpomenia claytoniae* was reported only from Ishigaki Island, Okinawa Prefecture (Boo *et al*. 2011). But, the species were found in Kyushu, Shikoku and Honshu in this study, suggesting its wider distributional range in Japan. *Colpomenia peregrina* had a wide distribution from Okinawa to Hokkaido, while *C*. *sinuosa* was found from Okinawa, Kyushu, Shikoku and Honshu. *Colpomenia borea* was identified molecularly only in samples from Akkeshi. But, it is also distributed in Muroran, based on herbarium specimens as mentioned in Chapter 2.

Conclusion

In conclusion, all phylogenetic trees reconstructed in this study are congruent and they revealed major evolutionary lineages of *Colpomenia* with highly supported branches for each species. Morphological characters were also consistent with each other. The genus *Colpomenia* showed to be a non-monophyletic. And thus, this genus is suitable for reclassification of genera by suggesting the *C*. *sinuosa* as entirely monophyletic *Colpomenia* clade. In addition, I believe that the four species, namely *C*. *claytoniae*, *C*. *peregrina*, *C*. *expansa*, and *C*. *borea*, under the monophyletic group should also be segregated away from *Colpomenia* by proposing a different genus and making several name combinations. As for the other species such as: *C. ecuticulata* and *C. ramosa*, they too should be considered for a different genus within the family Scytosiphonaceae.

Meanwhile, for *C*. *mollis* and *C*. *nainativensis*, due to the lack of samples for molecular analyses, short and partial fragments of DNA sequences available and limited morphoanatomical and life-history studies (both culture and field),

addressing these species are difficult at the moment. Maybe in future studies of other phycologists would make a full justification to provide concrete classification of these species.

Chapter 4.

Species delimitation of the genus *Colpomenia*

INTRODUCTION

Species delimitation and discrimination by morphology are difficult in many closely related taxa because of their morphological plasticity and simplicity or complexity depending on species (Hoshino *et al*. 2020). Molecular methods for species delimitation have been developed and used to investigate such closely related taxa including seaweed species (Song *et al*. 2019). In the Scytosiphonaceae, species of the genus *Scytosiphon* were investigated using DNA-based species delimitation methods. Since results of DNA-based species delimitation may differ depending on methods, multiple methods are recommended for accuracy of species delimitation (Kekkonen & Hebert 2014).

In Chapter 3, large variations of *cox*3 sequences were observed within each of *Colpomenia sinuosa*, *C. claytoniae*, and *C. peregrina*. In addition, the *cox*3 tree

showed subclades in each clade of *C*. *sinuosa* and *C*. *claytoniae*. These results may suggest cryptic species in these species.

In this chapter, DNA-based species delimitation was conducted for *Colpomenia* species, using *cox*3 gene sequences and four species delimitation methods.

Materials and methods

Methods from DNA extraction to molecular contig assembly in this chapter were done the same as in the previous chapter. Samples in this chapter were collected from around the coast of Japan, Russia, Portugal, and Australia (Figure 9 and Table 6) comprised of 151 *Colpomenia* sequences, both newly sequenced samples and downloaded sequences from GenBank (Table 5) and the same length of 593 base pairs.

All species delimitation analyses were performed online using four separate analyses: (1) the Automatic Barcode Gap Discovery or ABGD by (Puillandre *et al*. 2012) (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html), (2) the Assemble Species by Automatic Partitioning or ASAP method by (Puillandre *et al*. 2021) (https://bioinfo.mnhn.fr/abi/public/asap/), (3) Poisson Tree Processes or PTP, and (4) Bayesian Poisson Tree Processes or bPTP (Zhang *et al*. 2013) (https://species.h-its.org/ptp/). All available species under the genus *Colpomenia* were analyzed and all outgroup species were removed to avoid any confusion or problem while running these programs.

I used an aligned fasta as an input file for both the ABGD and ASAP analyses. They were run together using K80 ts/tv 2.0, since Kimura substitution model has been widely used in other studies (e.g., Hoshino *et al*. 2018), therefore this method was used to compute the distance, and all these remained under default value.

The ABGD was designed by Puillandre *et al*. (2012) to delimit species based on finding automatically the first intraspecific barcode gap, then it generates a primary partition where groups represent the first candidate species by dividing the data of the computed barcode gap, and to account mutation rate variation and overlaps between intra- and interspecific across species diversities. ABGD recursively applies the first two steps to each cluster of the primary partition. This recursion creates from primary to secondary partitions continually until no further splitting occur. The original ABGD tree groups can be found in Fig. 25.

A new species delimitation method by Puillandre *et al*. (2021) defined the ASAP algorithm an ascending hierarchical clustering and merging sequences into successful groups or species until they merged all sequences to form a single group. They indicated that this has steps wherein they assign a probability that quantifies the chances that each of its new groups is a single species, second, a computation of the between the previous and a new partition, and lastly these two probabilities and barcode gap width groups are then combined to be panmictic species and formed the most reliable species delimitation.

ASAP has several graphical outputs where it produces color-coded indicators depending on its probability of being a panmictic species at each node of the hierarchical clustering, and this color guides are easy to find whether some

nodes may be split into smaller groups or to navigate among partitions and choose the most relevant partition (Puillandre *et al*. 2021). Original ASAP groupings can be found in the Figs. 26a, b, c.

Both PTP and bPTP analyses were straightforward, robust, and fast. In these models, I ran and used RaxML as an input tree with same set parameters as in Chapter 3. These models can delimit species using non-ultrametric phylogenies because Zhang *et al*. (2013) modeled speciation rate by directly utilizing the number of substitutions (intra- and inter-specific branching rates). The input tree was unrooted, with 500000 MCMC number of generations, and 0.1 burn-in and 123 seed under default value. PTP uses maximum likelihood method wherein branch lengths were generated on two independent classes of Poisson processes: describes speciation rate substitution and within-species coalescent events. While bPTP is a support of the PTP, implemented in Bayesian inference providing posterior probabilities to delineate species. According to the authors, higher bootstrap node value shows all descendants from this node could be from one species. Thus, I set these criteria to maintain the integrity of each bootstrap node value: 'highly supported' if ≥ 1.0, 'strongly supported' if ≥ 0.8, and 'poorly supported' if < 0.6. And the original partition of PTP and bPTP can be found in Figs. 27a, b, c and 28a, b, c.

Results

In ABGD, it produced a prior maximum divergence of P= 1.00e-03 with a barcode gap distance of 0.046 as Partition 1, the smallest amongst all partitions generated. Recursive partition with 35 groups and initial partition with eight groups to partition the dataset. Since there were many groups in the recursive partition, it was doubtful. Meanwhile, the initial partition appeared to be an acceptable number of species groupings (Fig. 21): 'Group I', a total of 10 *Colpomenia borea* OTUs; 'Group II', included 46 *C*. *claytoniae* OTUs; 'Group III and IV' each had two *C*. *ecuticulata* OTUs and *C*. *expansa* OTUs; 'Group V' had the largest species, containing 68 *C*. *peregrina* OTUs; 'Group VI' contained only single *C*. *ramosa* OTU; and *C*. *sinuosa* has been grouped into two, 'Group VII' had 13 OTUs and nine OTUs in 'Group VIII' (Figs. 22, 23, 24).

ASAP data produced nine partitions (Fig. 21). A score of 3.000000 was the smallest value of primary species hypotheses in the respective partition. First group consisted of 10 *Colpomenia borea* OTUs; second group contains 46 OTUs of *C*. *claytoniae*; the third and fourth group each consisted of two OTUs in *C*. *ecuticulata* and *C*. *expansa,* respectively; fifth group had 68 *C*. *peregrina* OTUs; a single species, *C*. *ramosa*, in the sixth group; *C*. *sinuosa* was divided into three groups, seventh group had 12 OTUs, a single sequence in the eight group, and ninth group had nine OTUs (Figs. 22, 23, 24).

PTP and bPTP results suggested a greater number of species than those methods above, yielding 18 species. In both models, they were almost identical to each other (Fig. 21). Same groupings for PTP and bPTP were: 'Group I' was a highly supported *Colpomenia ramosa* OUT; 'Group II' had two highly supported *C*. *ecuticulata* OTUs. 'Group III' had 10 strongly supported *C*. *borea*

OTUs; two highly supported OTUs of *C*. *expansa* in 'Group IV'; and containing 68 poorly supported *C*. *peregrina* OTUs in 'Group V'.

Succeeding groupings from these analyses start to differ from each other (Figs. 22, 23, 24). In PTP, *Colpomenia sinuosa* had been from poorly supported to mostly high to strongly supported species. This species has at least nine groups, namely, 'Group VI' had 11; 'Groups VII, IX, XI, XIII, XIV, and XV' have one OTU; 'Group X' had four OTUs; and two OTUs in 'Group XII'. *C*. *claytoniae* was divided into four: 'Group VIII' had 17 strongly supported OTUs; 'Group XVI' had 26 poorly supported OTUs; highly supported 'Group XVII' had two OTUs; and 'Group XVIII' had one highly supported OTU.

In bPTP, *Colpomenia sinuosa* also had nine groups ranging from highly to strongly and poorly supported species. 'Group VII' had 10 OTUs; 'Groups VIII, XII, XIV, XVI, XVII, and XVIII' had a single OTU; 'Group XIII' has poorly supported four OTUs; and 'Group XV' had poorly supported two OTUs. And *C*. *claytoniae* was also divided into four groups with the same number of species as above but differed in group placements, 'Groups VI and IX' for bPTP. The last group in *C*. *claytoniae*, 'Group X' had two strongly supported OTUs, and 'Group XI' has a highly supported single OTU.

Discussion

Multiple markers are helpful and successful tool for delineating species to differentiate closely related species or separate individuals (Sites & Marshall 2003;

Leliaert *et al*. 2014; Geoffroy *et al*. 2015), and to know biogeographical locations or hidden diversity within each species. An example of this was conducted in previous chapter wherein the genus *Colpomenia* analyzed from all genes revealed notable lineages. And in this chapter, four species delimitation methods were conducted to evaluate such hypothesis in the Chapter 3.

All the species delimitation methods that I used showed at least some consistency in their partition. But, in ABGD results, recursive partition displayed large undulation and yielded 35 groups, an overestimation of putative species numbers. Such occurrence has been observed and confirmed by Puillandre *et al*. (2012), Ratnasingham & Herbert (2013), and Huang *et al*. (2020), where they pointed out that recursive partition is unstable and prone to excessive partitioning. While initial partition results are much more stable and consistent than recursive, as it yielded eight groups compared to 35 groups. Therefore, the recursive partition was not used in this study.

In previous chapter, Lineage I showed two clades of *Colpomenia sinuosa*. In the first clade of the ABGD, (Groups VII and VIII), ASAP (Groups VII to IX), PTP and bPTP (eleven groups), it represents the most diverse group, encompassing a range from cold-temperate to warm water. These are from the northern regions (Korea, Japan, France, Portugal, and USA), and at the southern regions (Australia, Kuwait, and South Africa). Species contained in ABGD Group VII were considered as one species. However, in ASAP, PTP and bPTP, these contained several species. Groups VI and VII of PTP and bPTP were from the northern parts. But in ASAP Group VII has mixed species both from north and south regions. Interestingly, ASAP (group VIII), PTP (groups VII, XIV, XV), bPTP (groups VII, XVII, XVIII), seem to represent

distinct species from the southern part. In addition, observing both PTP and bPTP groups showed strongly to highly supported species (0.8–1.0).

Lee *et al*. (2013) suggested an explanation why these groups of *C*. *sinuosa* species occurred in Australasia, East Asia, Europe, Middle East, and USA is because there is a possibility of recent gene flow and resulted from a long history of dispersal. This history of long dispersal remained uncertain, in contrast, several evidence about this could provide information.

In the second clade, ABGD group VIII and ASAP group IX, *Colpomenia sinuosa* was partitioned similarly. OTUs of these groups were from the Indo-Pacific tropical to warm waters, from Okinawa, Japan and Taiwan to Australia and the Philippines. But PTP and bPTP yielded five groups in this clade. The more number of groups in PTP and bPTP seems to be an over estimate. However, the OTUs of groups seem to be related to their collection locality. Further studies are required using more samples from more localities to reveal species boundaries in *C*. *sinuosa*.

The Lineage III consisted of *Colpomenia ecuticulata*. This species has two sequences and showed consistent independent species from all delimitation analyses. This species is distributed in Pakistan, Korea, Australia, New Zealand, and islands in the Pacific. Although it showed a distinct clade, additional samples of this species should be collected and sequenced.

In Chapter 3, Lineage II showed a monophyletic clade which were composed of four species namely, *Colpomenia borea*, C. *claytoniae*, *C*. *expansa* and *C*. *peregrina*.

*Cox*3 sequence differences within *Colpomenia borea* were small, and a single species was suggested by all the species delimitation analyses.

The clade of *C. claytoniae* had four diverse groups (Figure 23) in PTP and bPTP. These four groups: X, XI, XVII, and XVIII from Korea, Japan, and Australia, exhibit high supports up to 1.0. The group XVI has 0.6 value, meaning poorly supported species group. While in ABGD or ASAP, this species was considered as a single species. However, this group may be a diverse and environmental tolerant group, where they can be found from eight geographic locations around the world, ranging from cold to warm waters from Australia, Hong Kong, Japan, Korea, New Zealand, Portugal, USA, and South Africa. Although this study could not reveal whether there are cryptic species in *C. claytoniae*, two or more cryptic species may be included in this species.

Third in the lineage is *Colpomenia expansa*, with two samples available for analysis. The *cox*3 sequence differences between the two samples was small. These two samples were from Korea, but this species has been reported from North America, Mexico, and Korea. All species delimitation analyses resulted in a single species. But more samples from its distribution regions are needed for further studies.

Across all delimitation models, *Colpomenia peregrina* produced a single big partition, as shown similarly in the *cox*3 BI and ML tree from the previous chapter. In PTP and bPTP results showed poor support of only 0.6, thus may be due to multiple species contained here. *Colpomenia peregrina* is commonly found and has been reported from the areas in Northwest Pacific (Japan, Korea, Russia), Southwest Pacific (Australia, New Zealand), Northeast Pacific (United States, Mexico), and Atlantic (France, Norway, Ireland, United Kingdom, Spain, United States), but absent in South America and South Africa. The distributional pattern suggests that the evolution and putative center of the diversity of *C.*

peregrina was in the Northwest Pacific (Clayton 1975; Parsons 1982; Ramírez & Rojas 1991; Kogame & Yamagishi 1997; Stegenga *et al*. 1997; Cho *et al*. 2005). This was confirmed by Lee *et al*.'s (2014a) study using the *cox*3 gene, and *C*. *peregrina* were likely originated in this region. They also suggested that this area has a subsequent pre-LGM (beginning ca. 50 kya, several glacial/interglacial cycles prior to the Last Glacial Maximum, 30–22 kya, noticeable acceleration around 20kya (Bradwell *et al*. 2008)). Additionally, oyster cultures from Japan were done probably of *C*. *peregrina*'s repeated global dispersals (Luning 1990).

Lastly, the Lineage IV consisted of *Colpomenia ramosa* was still seen as a separated clade. However, this species has provided only one sequence available, but based on the partitioning, it is a highly supported species with a value of 1.0. This species can be found in Central and South America; however, their diversity around the world remained unanswered.

Conclusion

Boundaries of species were not fully corresponded among results of the four DNA-based species delimitation analyses. But species boundaries that corresponded among all species delimitation analyses supported the current classification except for *C*. *sinuosa* which was divided into two by the boundary. *Colpomenia sinuosa* could include at least two species.

To better understand their boundaries, more sequences should be added in the future. In addition, the integration of different species delimitation software

would enable us to address and provide more insights, such as species boundaries, the genetic evolutionary trajectory over time, and global distribution.

Although these data showed almost similar results, there is still an immediate need for conducting more analysis for the large number of putative species to provide more accurate representation of species partitioning. Furthermore, this chapter's investigations might initially and at least partially resolve and understand the taxonomy and species boundaries of the genus *Colpomenia*.

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FIGURES

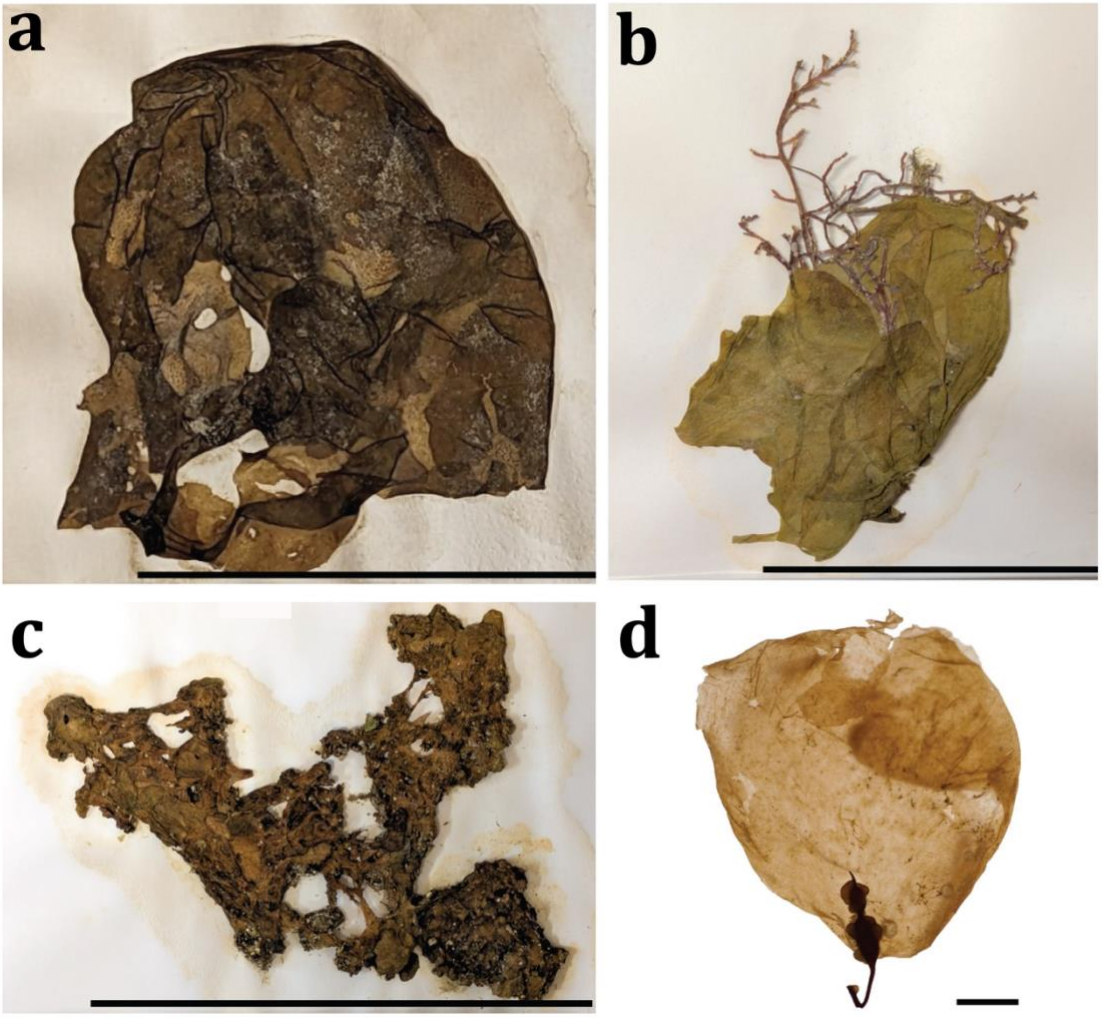

Figure 1. Representative species of *Colpomenia*. **a.** *C. sinuosa* collected at Senaga, Tomigusuku, Okinawa, Japan on 2009 March 30. Scale bar = 6 cm; **b.** *C. peregrina* collected at Oshoro, Otaru, Hokkaido, Japan on 2017 April 28. Scale bar = 10 cm; **c.** *C. claytoniae* collected at Amarube, Kazumiku, Hyogo, Japan on 2012 April 23. Scale bar = 12 cm; **d.** *C. borea* collected at Akkeshi, Hokkaido, Japan on 2013 June 10. Scale bar = 5 cm.

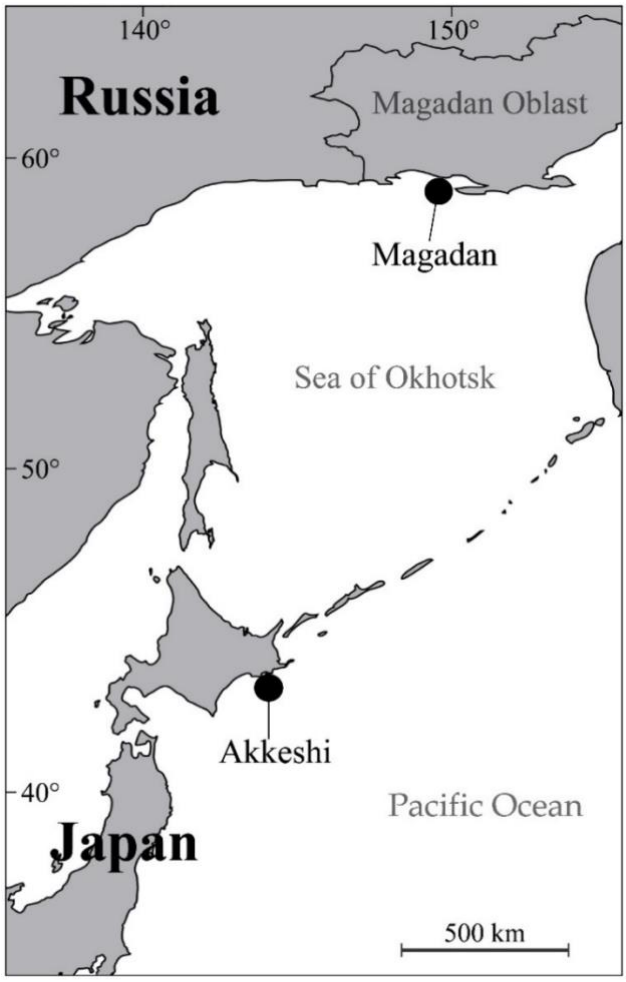

Figure 2. Map of collection localities of *Colpomenia borea* sp. nov.

Figure 3. Habitat of *Colpomenia borea* sp. nov. in Akkeshi, Hokkaido, Japan.

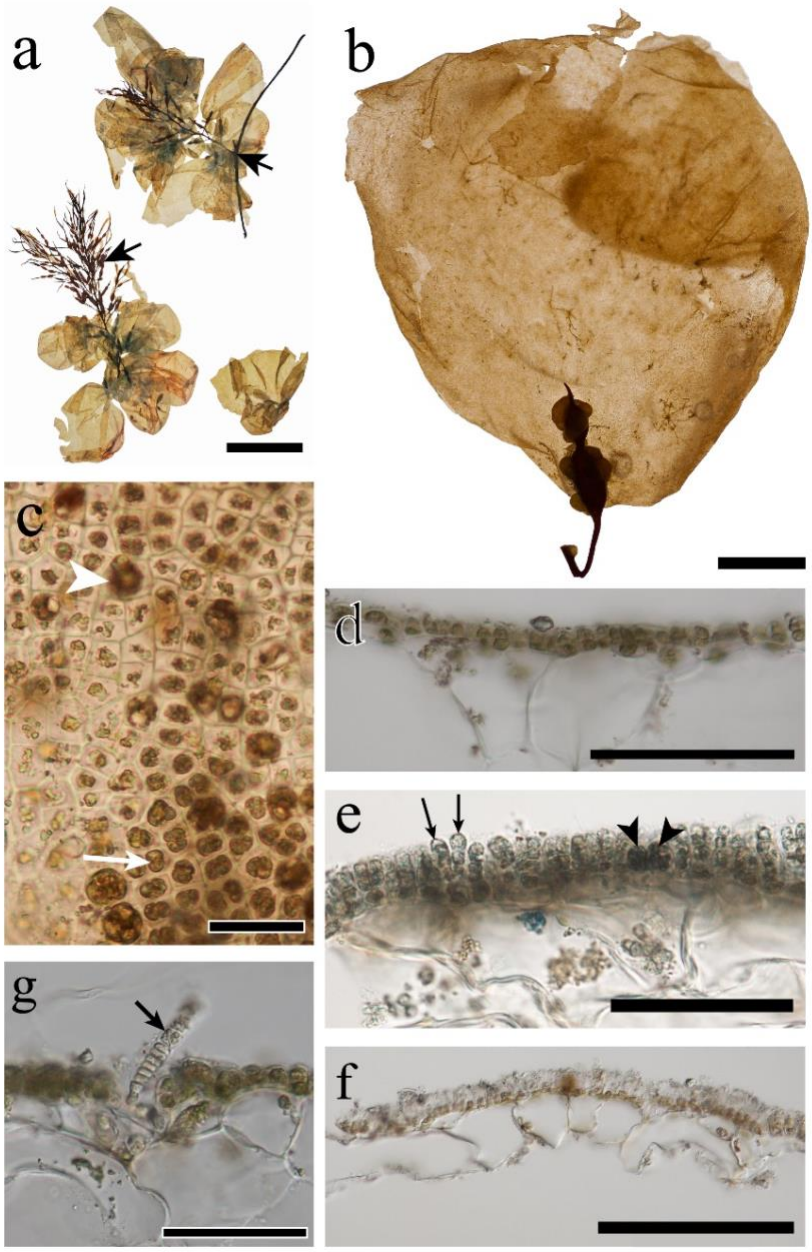

Figure 4. Habit and morphology of *Colpomenia borea* sp. nov.

(a) Holotype specimen of *Colpomenia borea* (SAP115473) attached on Stephanocystis crassipes (arrowheads). Scale bar = 2 cm. (b) Rehydrated salt-preserved sample of *Colpomenia borea*. Scale bar = 5 mm. (c) Surface view of the ascocyst (arrowhead) and young plurilocular sporangia (putative gametangia) (arrow). Scale bar = 30 µm. (d) Cross section of a thallus showing the pigmented cortex, small polygonal shape cells. Scale bar = 100 µm. (e) Cross section showing the putative gametangia (arrows) and ascocysts (arrow heads). Scale bar = 70 µm. (f) Section of the thin colorless polygonal medullary cells. Scale bar = 100 µm. (g) Phaeophycean hair (arrow) arising from the medullary cells. Scale bar = 100 µm.

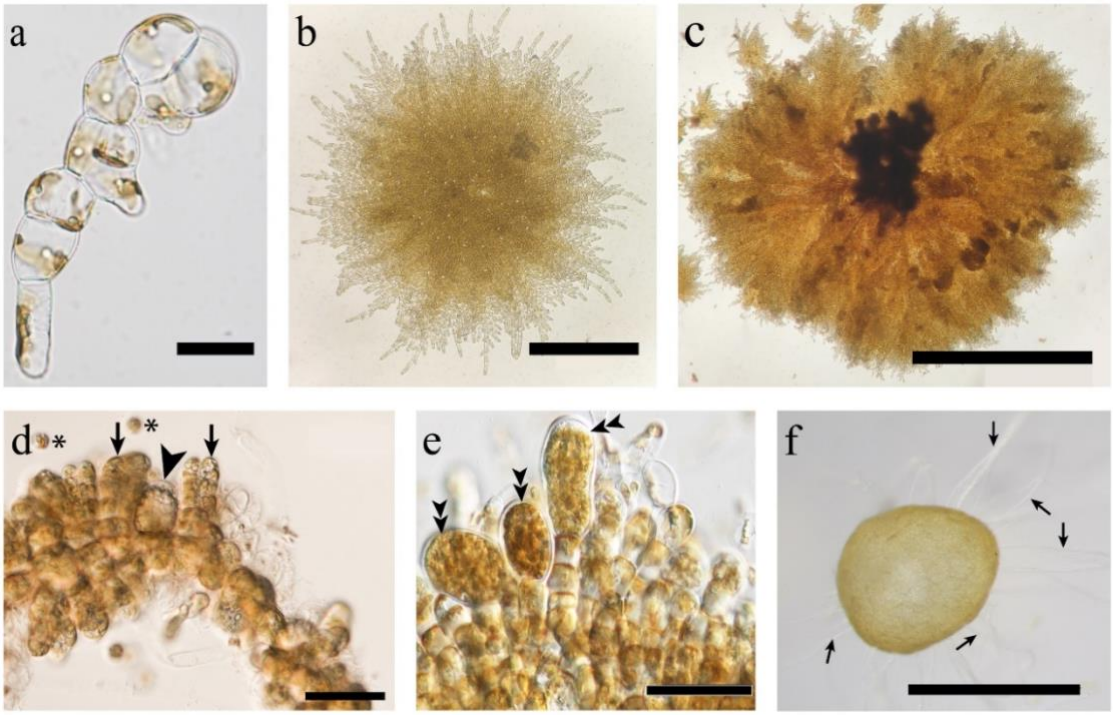

Figure 5. Culture of *Colpomenia borea*.

(a) Seven-day old germling of a plurispore (putative gamete) in a long-day condition at 15°C. Scale bar = 25 μm. (b) Pseudodiscoid thallus in the early stage. Scale bar = 0.5 mm. (c) Pseudodiscoid thallus when mature. Scale bar = 5 mm. (d) Squashed pseudodiscoid thallus grown in a long-day condition at 15°C, showing plurilocular sporangium (arrows), ascocysts (arrowhead) and plurispores (asterisks). Scale bar = 40 μm. (e) Squashed pseudodiscoid thallus grown in a short-day condition at 10°C, showing unilocular sporangia (double arrowheads). Scale bar = 50 μm. (f) Small saccate thalli which developed from a unispore in a short-day condition at 10°C. Arrows indicate phaeophycean hairs. Scale bar = 1 mm.

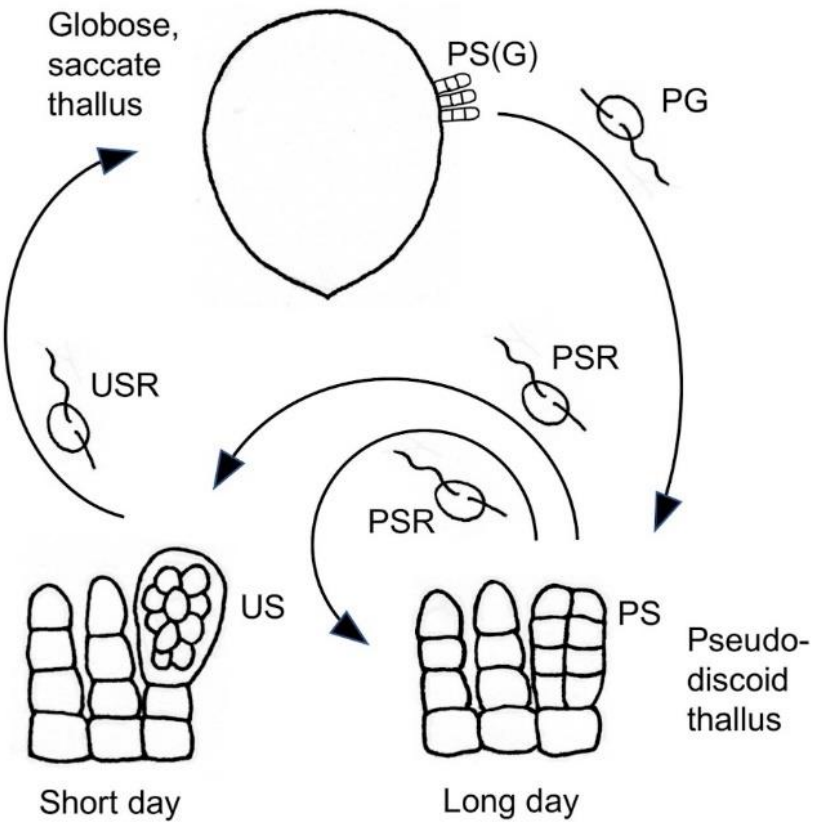

Figure 6. A diagram of a life history of *Colpomenia borea* in culture.

In pseudodiscoid thalli (sporophytic thalli), plurilocular sporangia were formed in a long-day condition, and unilocular sporangia were formed in a short-day condition. PS(G), putative gametanigum; PG, putative gamete; PS, plurilocular sporangium; PSR, plurispore released from plurilocular sporangium; US, unilocular sporangium; USR, unispore released from unilocular sporangium.

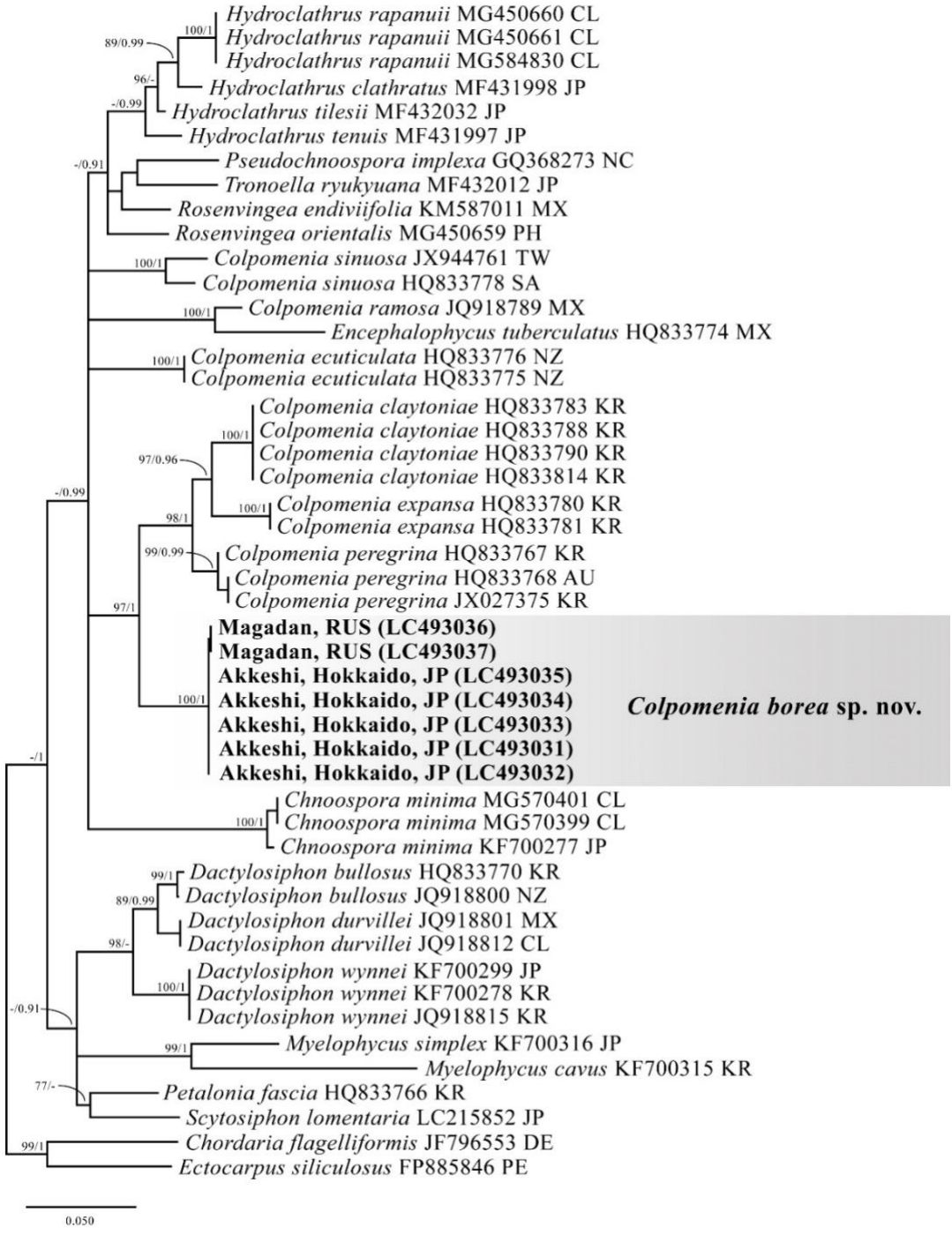

Figure 7. Maximum likelihood (ML) tree of *Colpomenia* inferred from *cox*3 sequences. Values above the branches are maximum likelihood bootstrap values (BP) and Bayesian inference posterior probabilities (PP) (BP/PP, BP>70, PP>0.95). AU, Australia; CL, Chile; DE, Germany; JP, Japan; KR, Korea; MX, Mexico; NC, New Caledonia; NZ, New Zealand; PE, Peru; PH, Philippines; RUS, Russia; ZA, South Africa; TW, Taiwan.

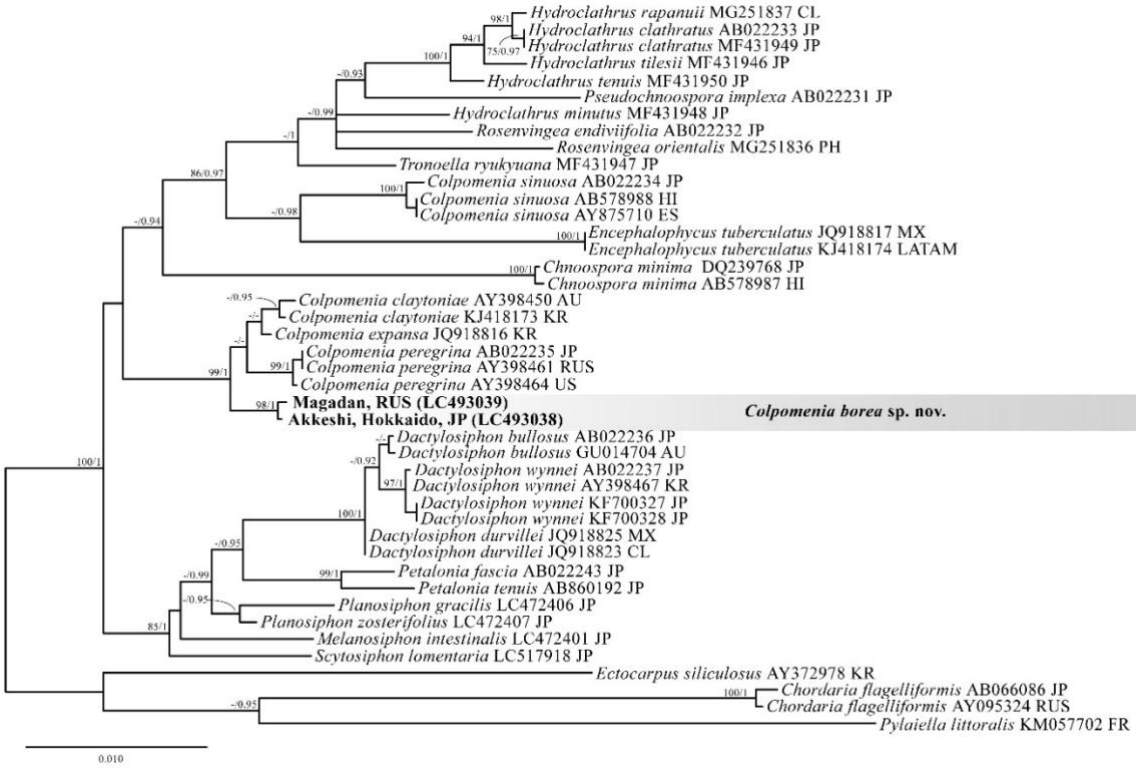

Figure 8. Maximum likelihood (ML) tree of *Colpomenia* inferred from *rbc*L sequences. Values above the branches are maximum likelihood bootstrap values (BP) and Bayesian inference posterior probabilities (PP) (BP/PP, BP>70, PP>0.95). AU, Australia; CL, Chile; FR, France; HI, Hawaii; JP, Japan; KR, Korea; LATAM, Latin America; MX, Mexico; PH, Philippines; RUS, Russia; ES, Spain; US, United States of America.

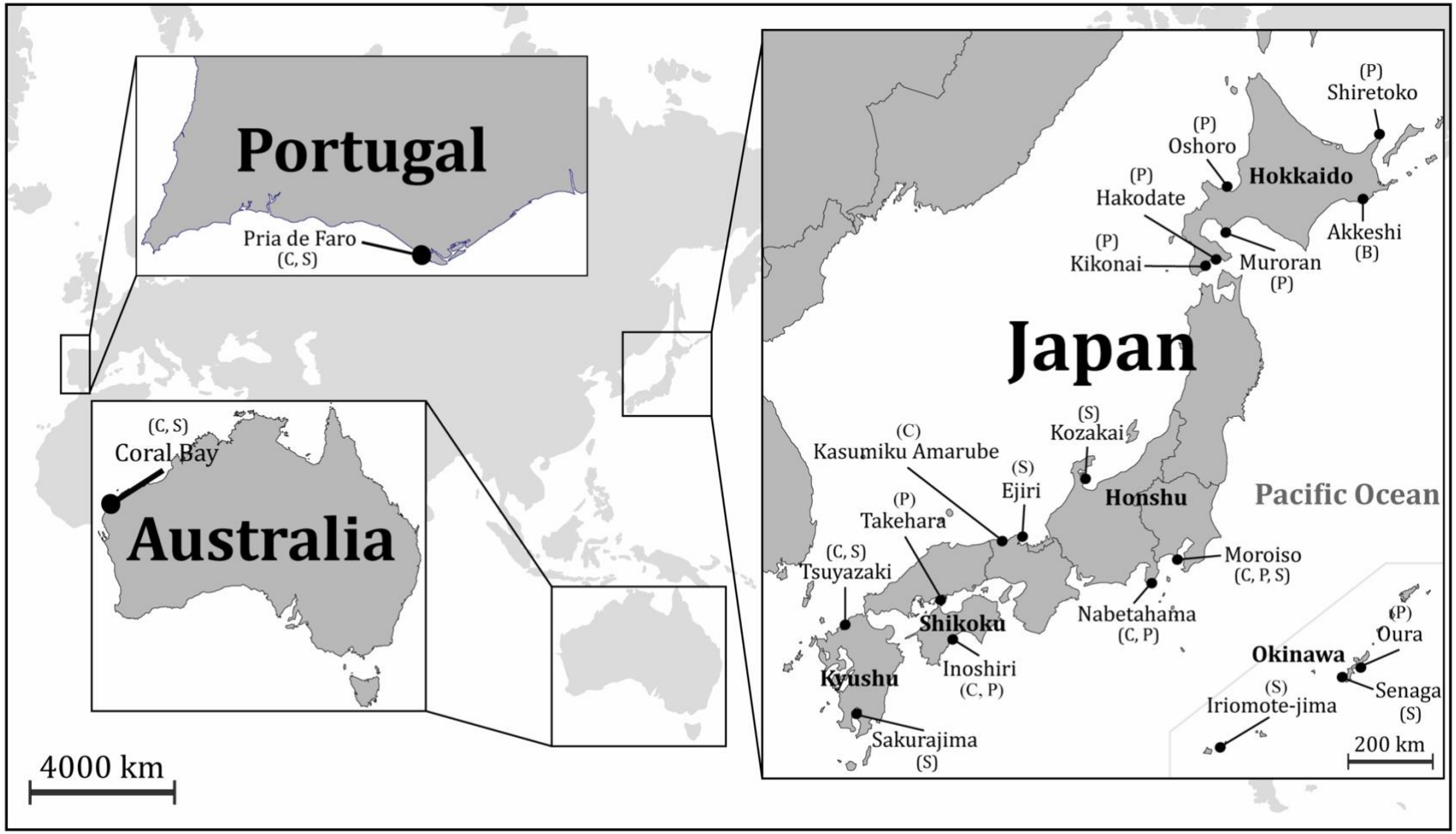

Figure 9. Collection localities in Japan, Australia, and Portugal. Letters above or below the locality names correspond to the *Colpomenia* species found in these areas. (B) *C. borea*; (C) *C. claytoniae*; (P) *C. peregrina*; and (S) *C. sinuosa*.

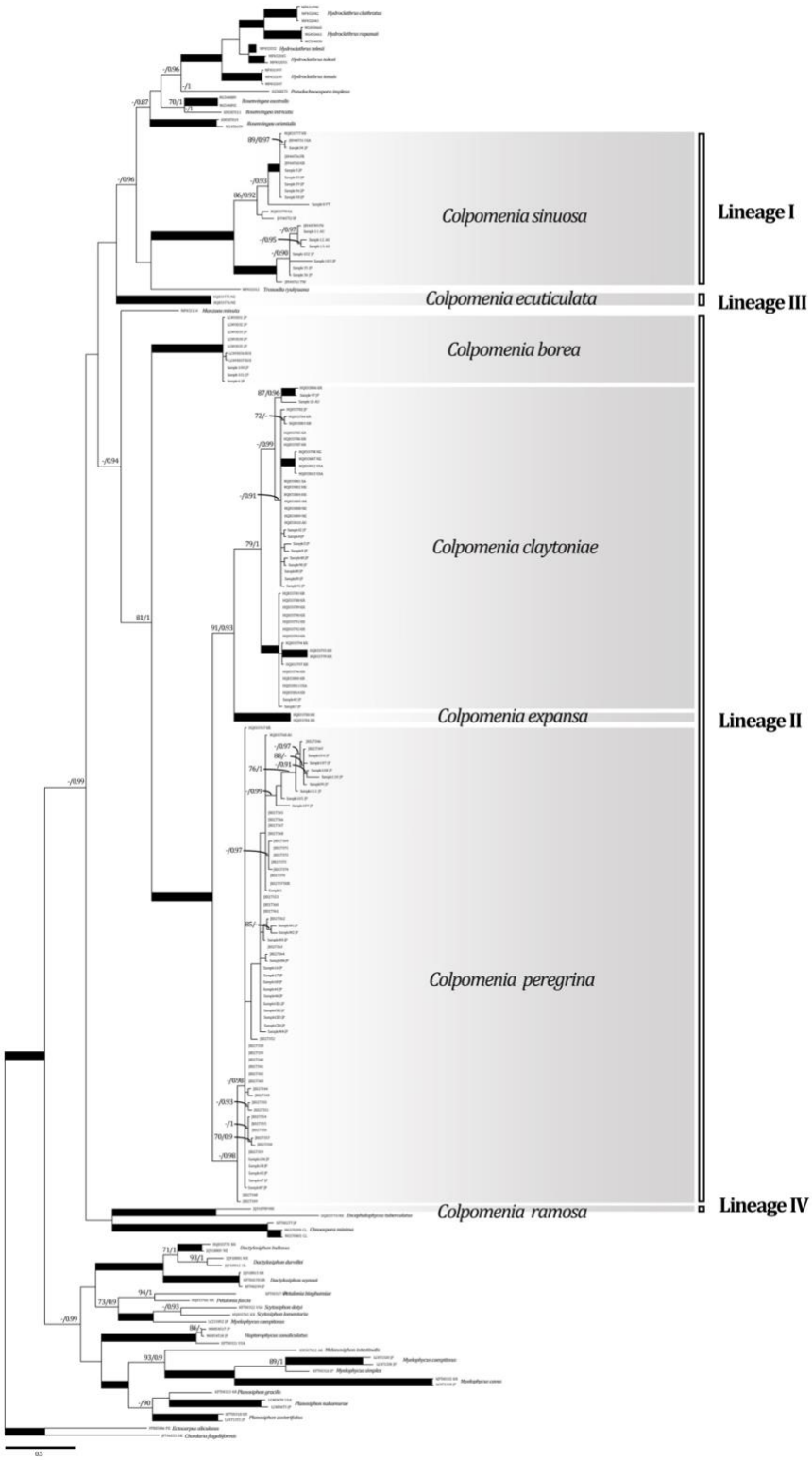

Figure 10. Maximum likelihood (ML) tree of *Colpomenia* inferred from *cox*3 sequence data. Values above or below the branches are maximum likelihood bootstrap values (BP) and Bayesian inference posterior probabilities (PP). Thickened lines indicate highly supported branches (BP: ≥ 95 % and PP: ≥ 0.98). Poorly supported values (BP < 70% and PP < 0.90) are removed.

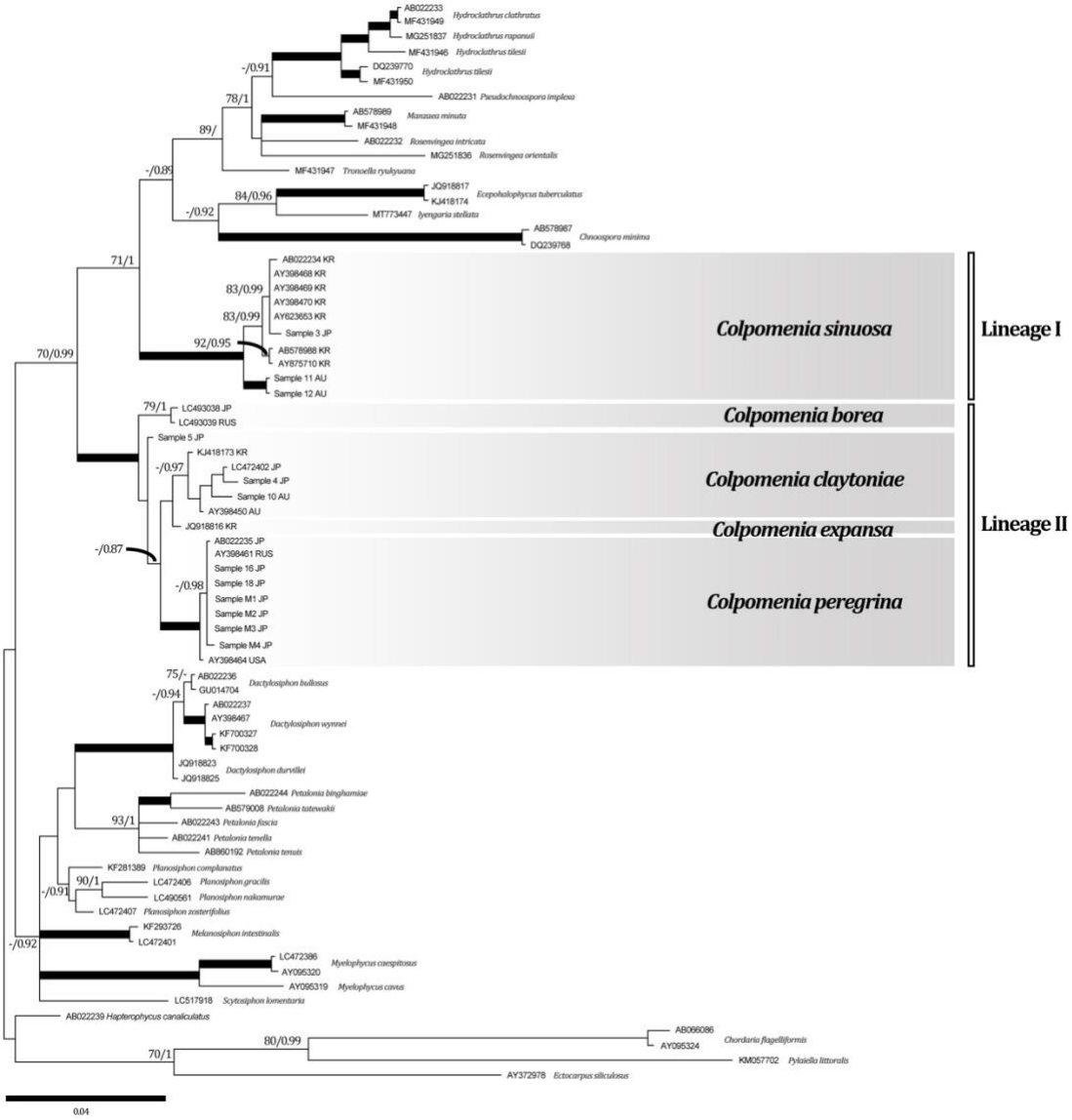

Figure 11. Maximum likelihood (ML) tree of *Colpomenia* inferred from *rbc*L sequence data. Values above or below the branches are maximum likelihood bootstrap values (BP) and Bayesian inference posterior probabilities (PP). Thickened lines indicate highly supported branches (BP: ≥ 95 % and PP: ≥ 0.98). Poorly supported values (BP < 70% and PP < 0.90) are removed.

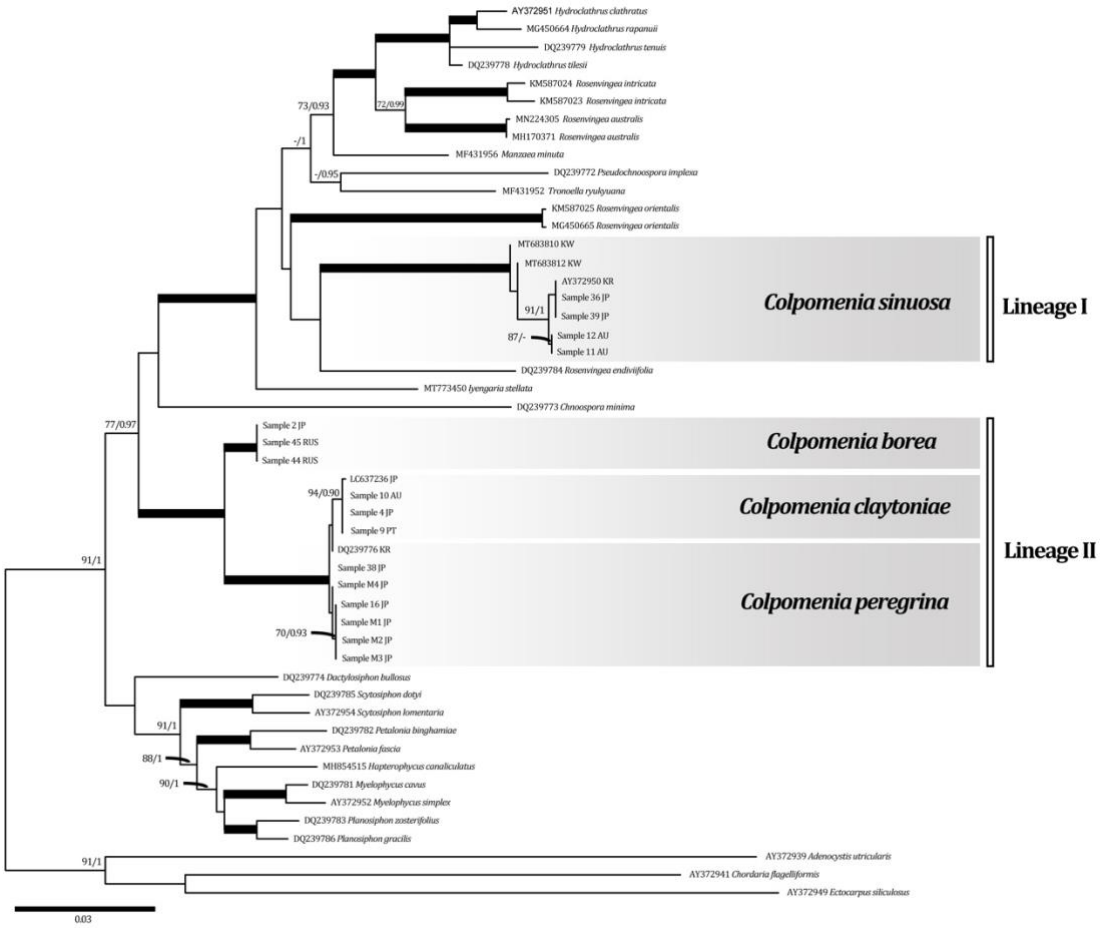

Figure 12. Maximum likelihood (ML) tree of *Colpomenia* inferred from *psa*A sequence data. Values above the branches are maximum likelihood bootstrap values (BP) and Bayesian inference posterior probabilities (PP). Thickened lines indicate highly supported branches (BP: ≥ 95 % and PP: ≥ 0.98). Poorly supported values (BP: < 70% and PP: < 0.90) are removed.

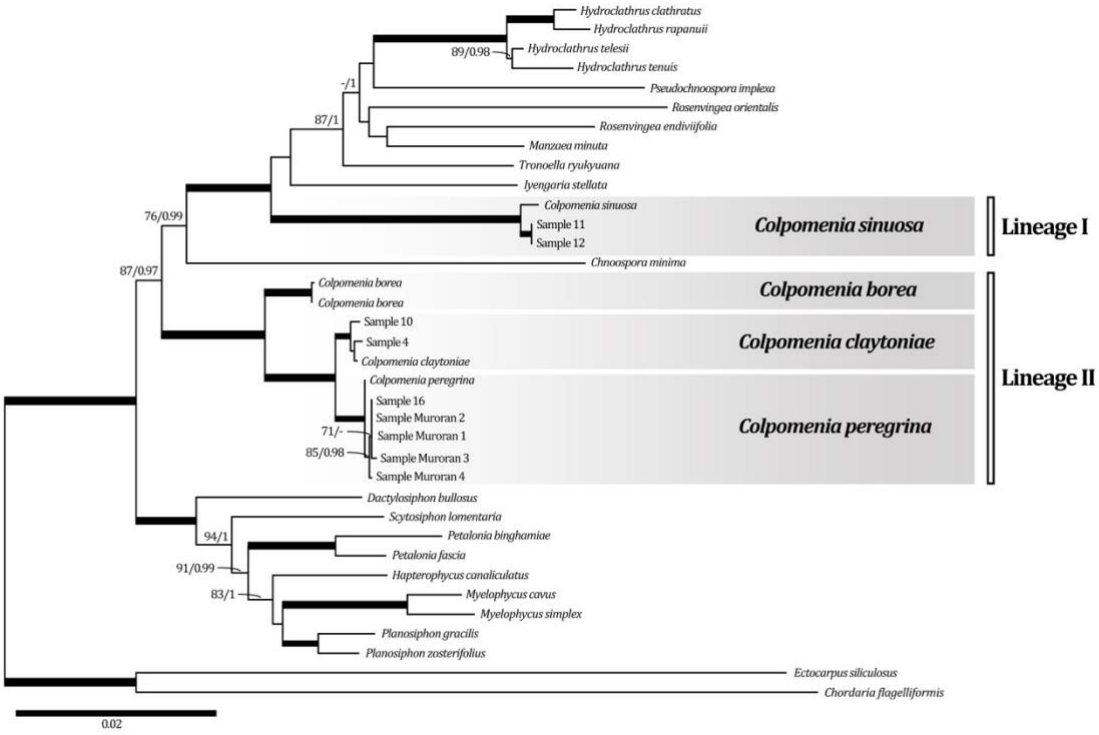

Figure 13. Concatenated (*rbc*L+*psa*A) maximum likelihood (ML) tree of *Colpomenia* sequence data. Values above the branches are maximum likelihood bootstrap values (BP) and Bayesian inference posterior probabilities (PP). Thickened lines indicate highly supported branches (BP: ≥ 95 % and PP: ≥ 0.98). Poorly supported values (BP < 70% and PP < 0.90) are removed.

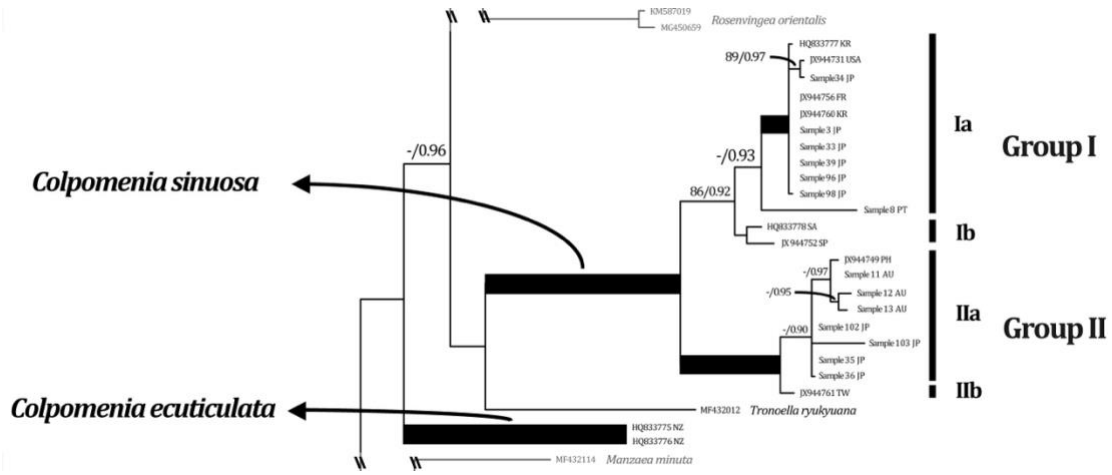

Figure 14. Detailed *cox*3 maximum likelihood (ML) tree of *C*. *sinuosa* and *C*. *ecuticulata* grouping. Values above the branches are maximum likelihood bootstrap values (BP) and Bayesian inference posterior probabilities (PP). Thickened lines indicate highly supported branches (BP: ≥ 95 % and PP: ≥ 0.98). Poorly supported values (BP < 70% and PP < 0.90) are removed.

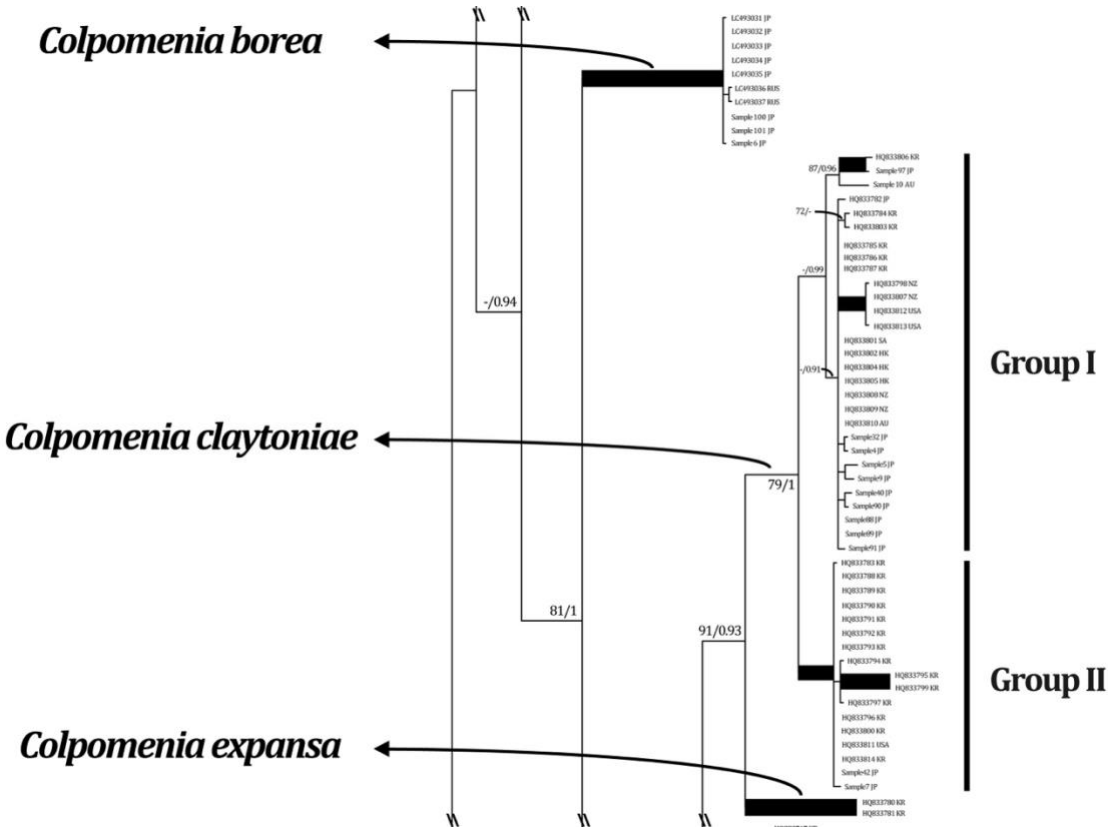

Figure 15. Detailed *cox*3 maximum likelihood (ML) tree of *C*. *borea*, *C*. *claytoniae*, and *C*. *expansa* grouping. Values above the branches are maximum likelihood bootstrap values (BP) and Bayesian inference posterior probabilities (PP). Thickened lines indicate highly supported branches (BP: ≥ 95 % and PP: ≥ 0.98). Poorly supported values (BP < 70% and PP < 0.90) are removed.

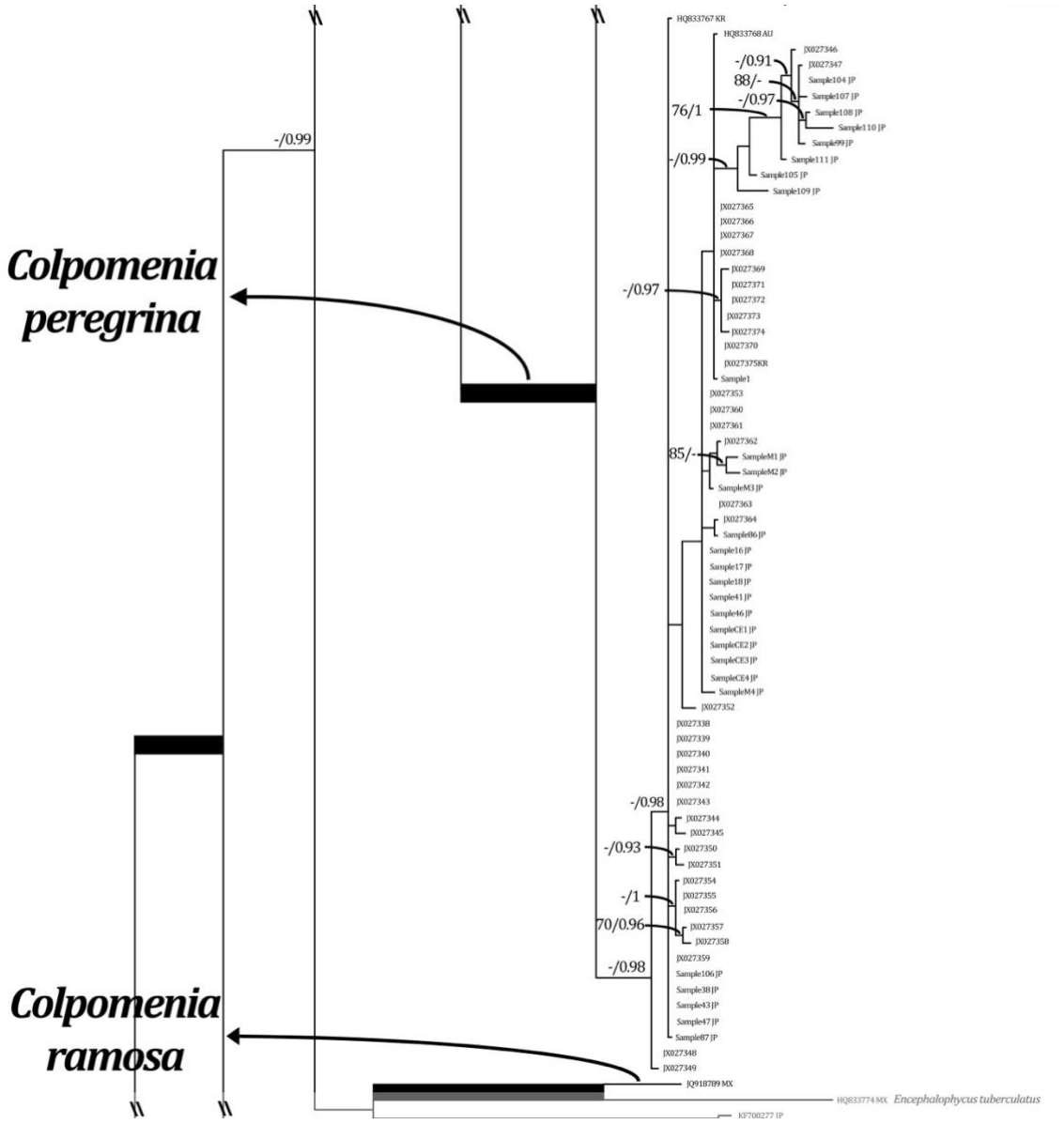

Figure 16. Detailed *cox*3 maximum likelihood (ML) tree of *C. peregrina* and *C. ramosa* grouping. Values above the branches are maximum likelihood bootstrap values (BP) and Bayesian inference posterior probabilities (PP). Thickened lines indicate highly supported branches (BP: ≥ 95 % and PP: ≥ 0.98). Poorly supported values (BP < 70% and PP < 0.90) are removed.

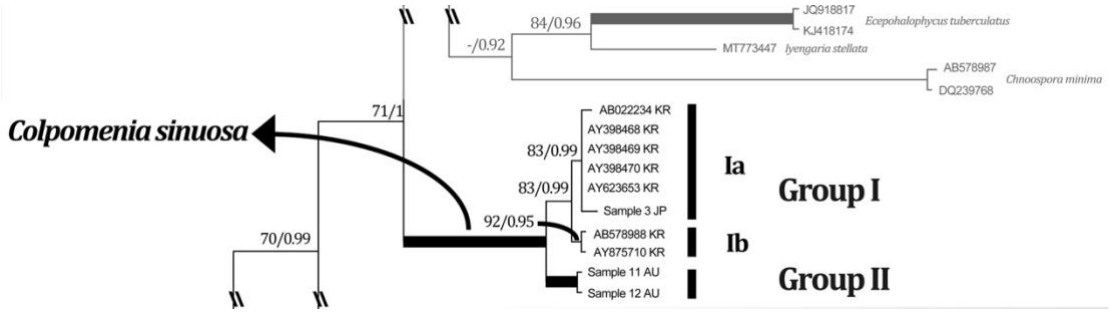

Figure 17. Detailed *rbc*L maximum likelihood (ML) tree of *C. sinuosa* grouping. Values above the branches are maximum likelihood bootstrap values (BP) and Bayesian inference posterior probabilities (PP). Thickened lines indicate highly supported branches (BP: ≥ 95 % and PP: ≥ 0.98). Poorly supported values (BP < 70% and PP < 0.90) are removed.

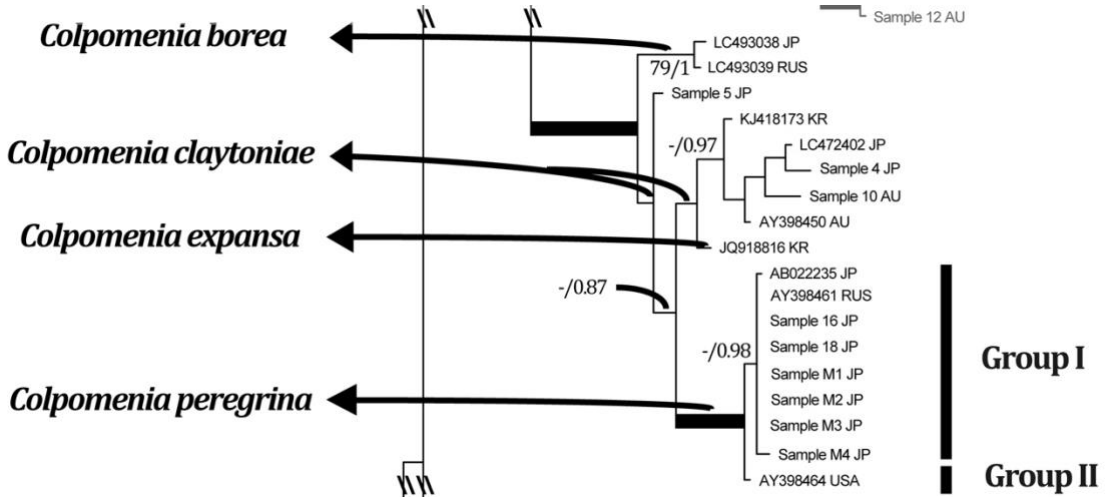

Figure 18. Detailed *rbc*L maximum likelihood (ML) tree of *C. borea*, *C. claytoniae*, *C. expansa*, and *C. peregrina* grouping. Values above the branches are maximum likelihood bootstrap values (BP) and Bayesian inference posterior probabilities (PP). Thickened lines indicate highly supported branches (BP: ≥ 95 % and PP: ≥ 0.98). Poorly supported values (BP < 70% and PP < 0.90) are removed.

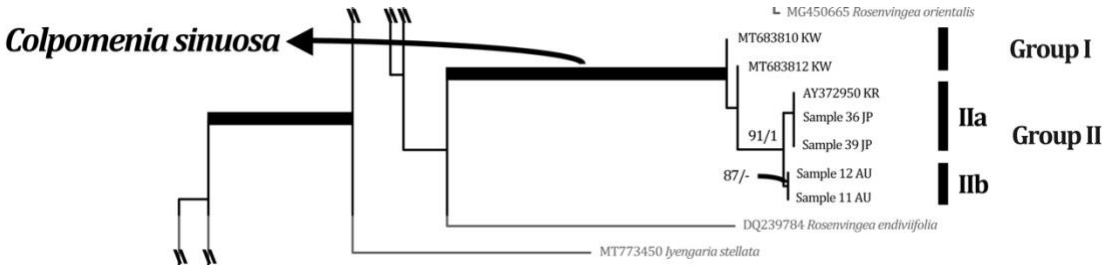

Figure 19. Detailed *psa*A maximum likelihood (ML) tree of *Colpomenia sinuosa* grouping. Values above the branches are maximum likelihood bootstrap values (BP) and Bayesian inference posterior probabilities (PP). Thickened lines indicate highly supported branches (BP: ≥ 95 % and PP: ≥ 0.98). Poorly supported values (BP < 70% and PP < 0.90) are removed.

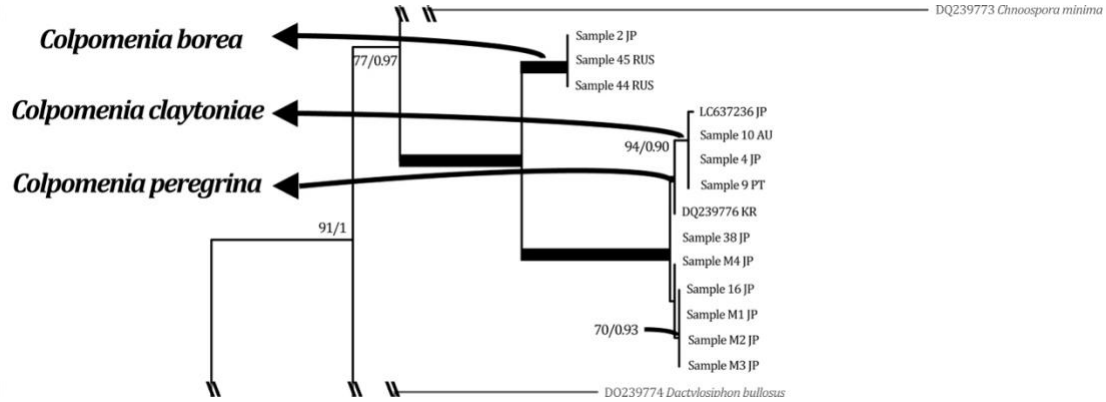

Figure 20. Detailed *psa*A maximum likelihood (ML) tree of *C. borea*, *C. peregrina*, and *C. claytoniae* grouping. Values above the branches are maximum likelihood bootstrap values (BP) and Bayesian inference posterior probabilities (PP). Thickened lines indicate highly supported branches (BP: ≥ 95 % and PP: ≥ 0.98). Poorly supported values (BP < 70% and PP < 0.90) are removed.

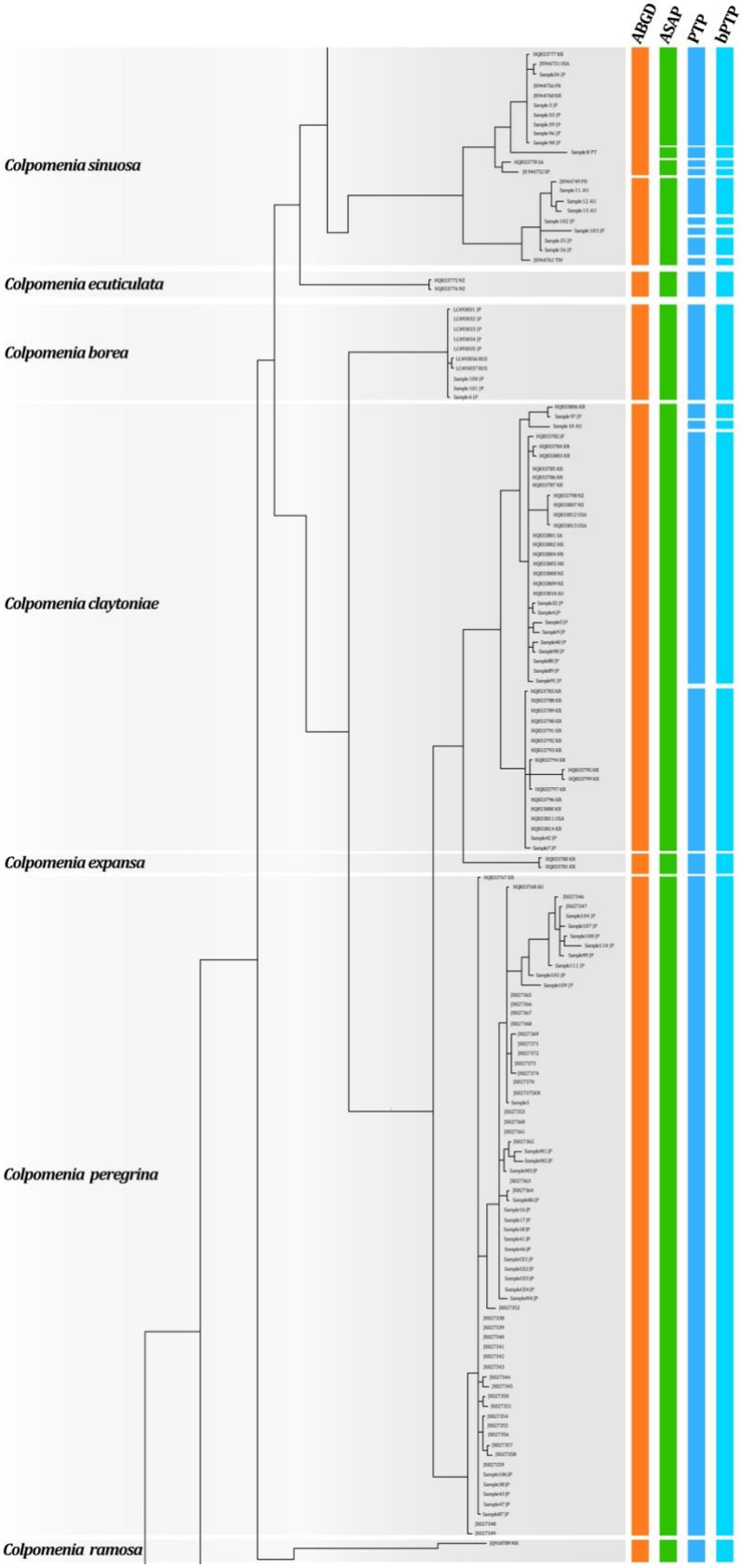

Figure 21. Maximum Likelihood tree based on *cox*3 within the genus *Colpomenia*. The results of species delimitation groupings (ABGD, ASAP, PTP, and bPTP) were indicated in different colors.

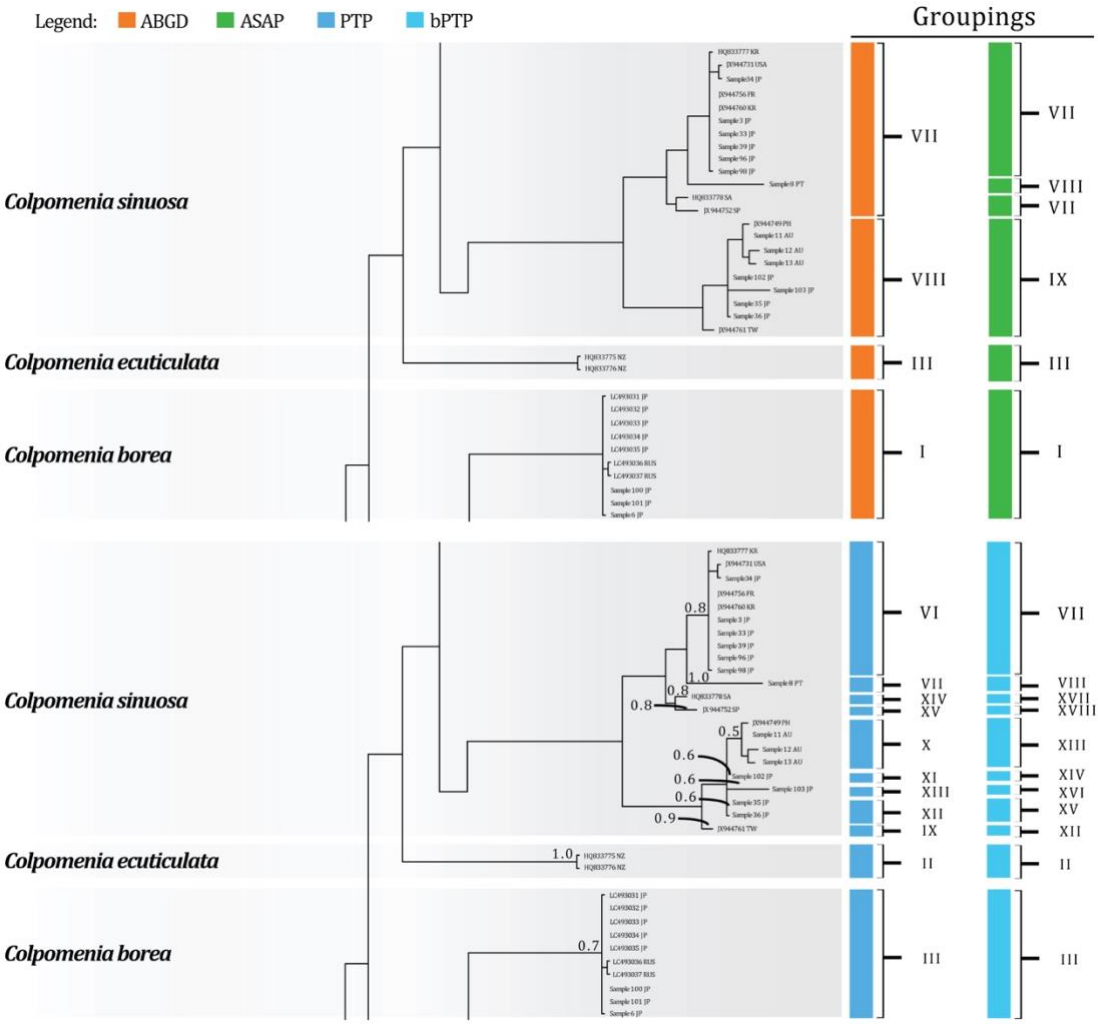

Figure 22. Groupings of *Colpomenia sinuosa, C. ecuticulata*, and *C. borea* partitioned by ABGD, ASAP, PTP, and bPTP analyses were written in roman numeral. Values on or under the branch indicates support.

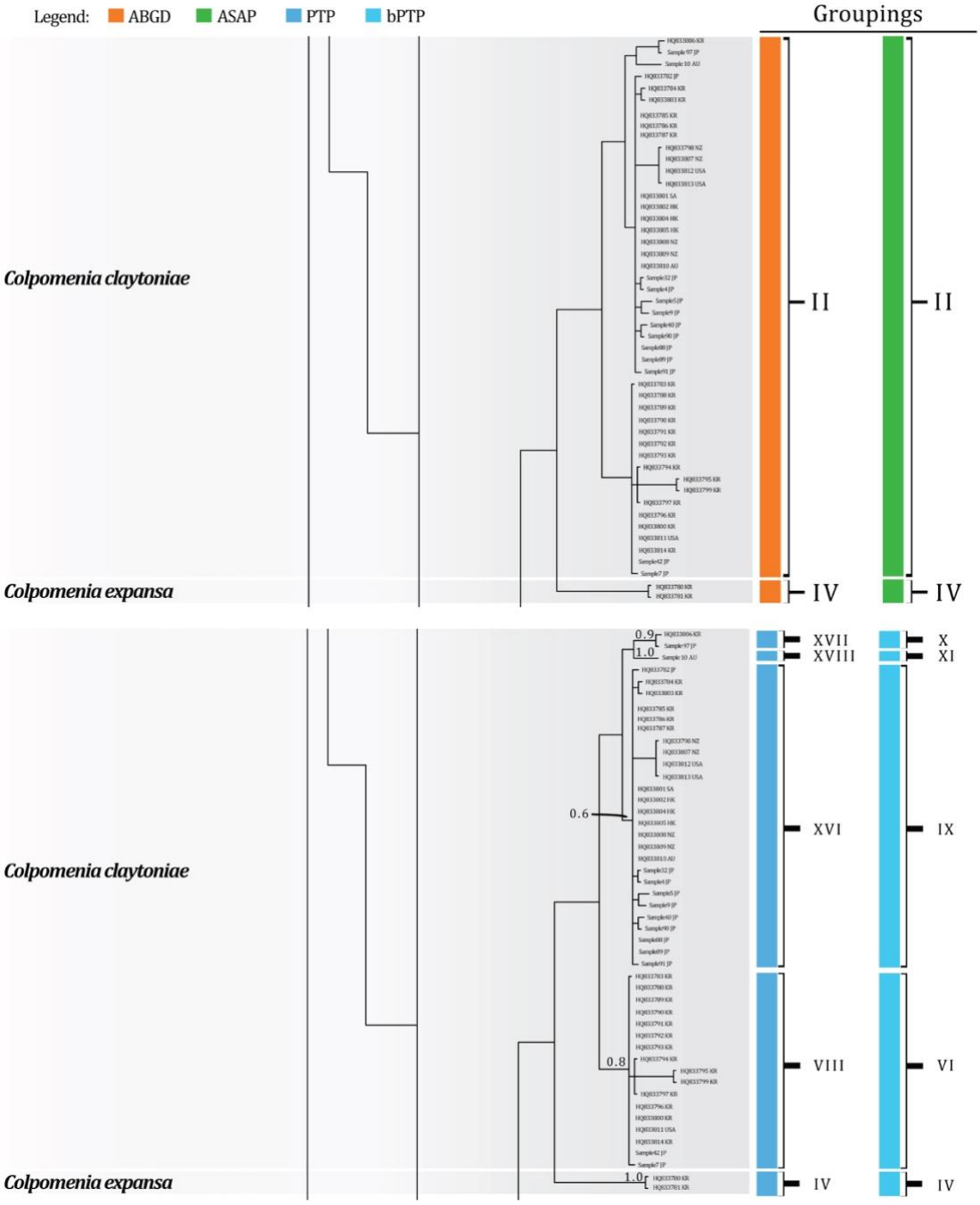

Figure 23. Groupings of *Colpomenia claytoniae* and *C. expansa* partitioned by ABGD, ASAP, PTP, and bPTP analyses were written in roman numeral. Values on or under the branch indicates support.

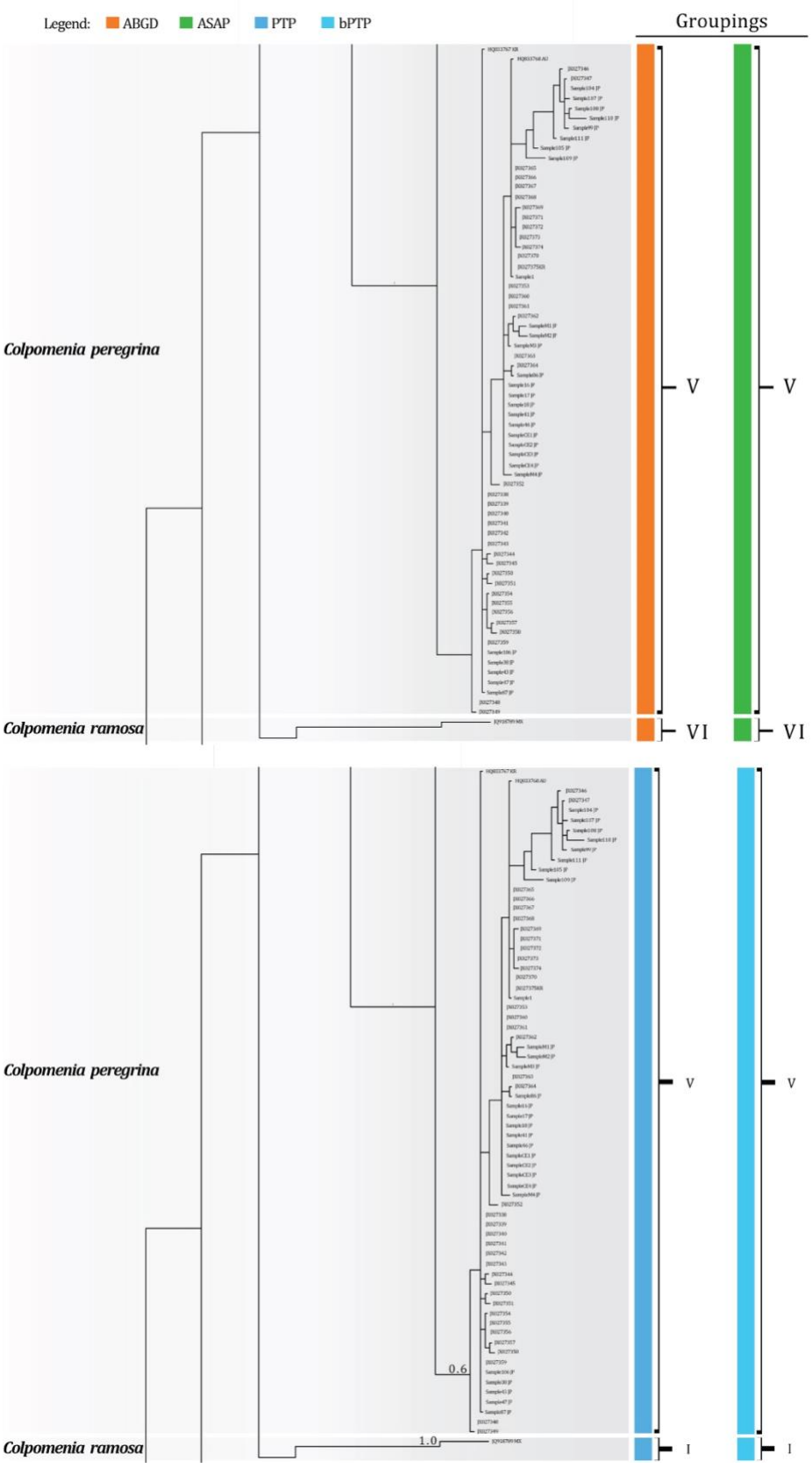

Figure 24. Groupings of *Colpomenia peregrina* and *C. ramosa* partitioned by ABGD, ASAP, PTP, and bPTP analyses were written in roman numeral. Values on or under the branch indicates support.

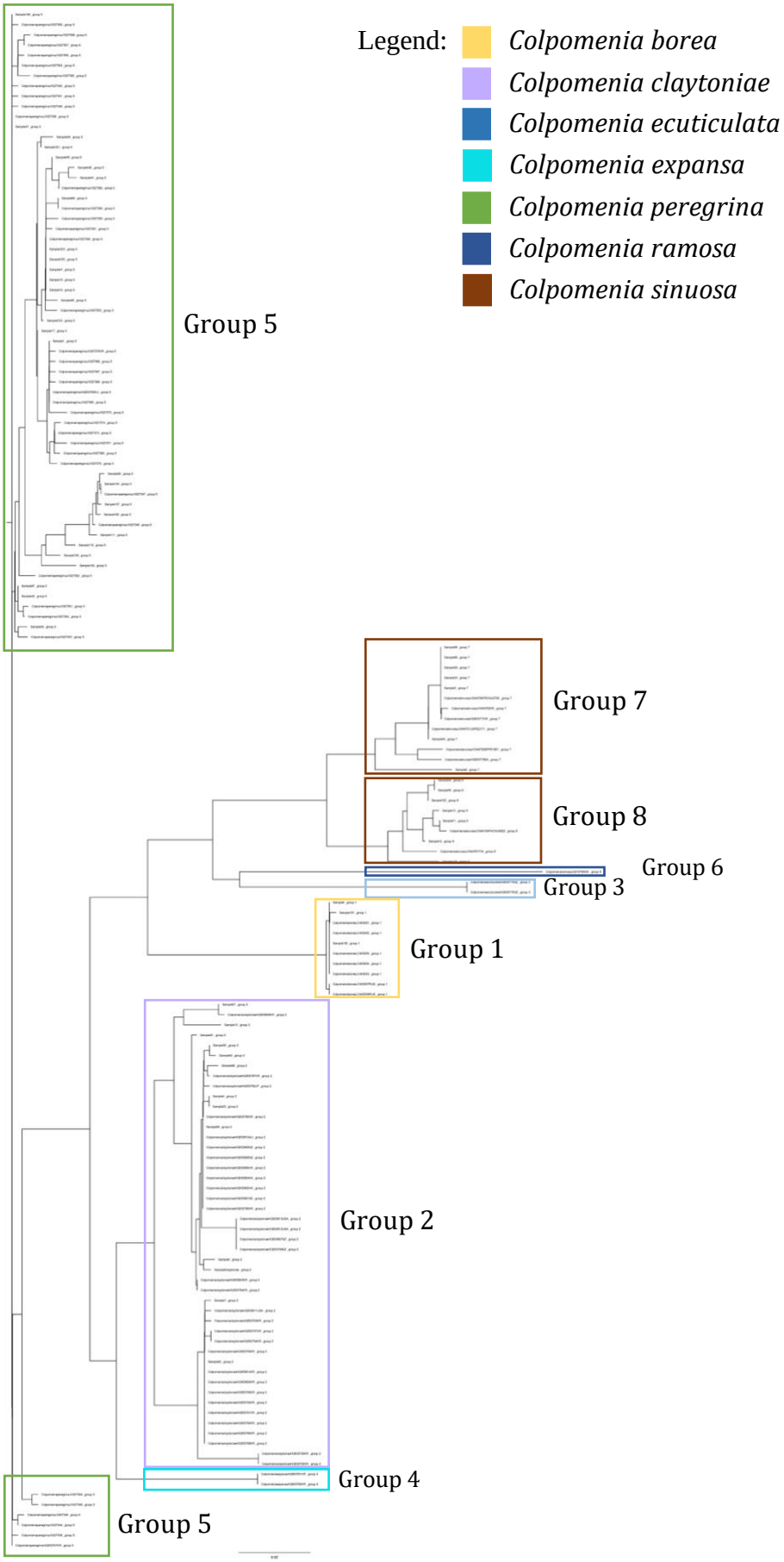

Figure 25. ABGD

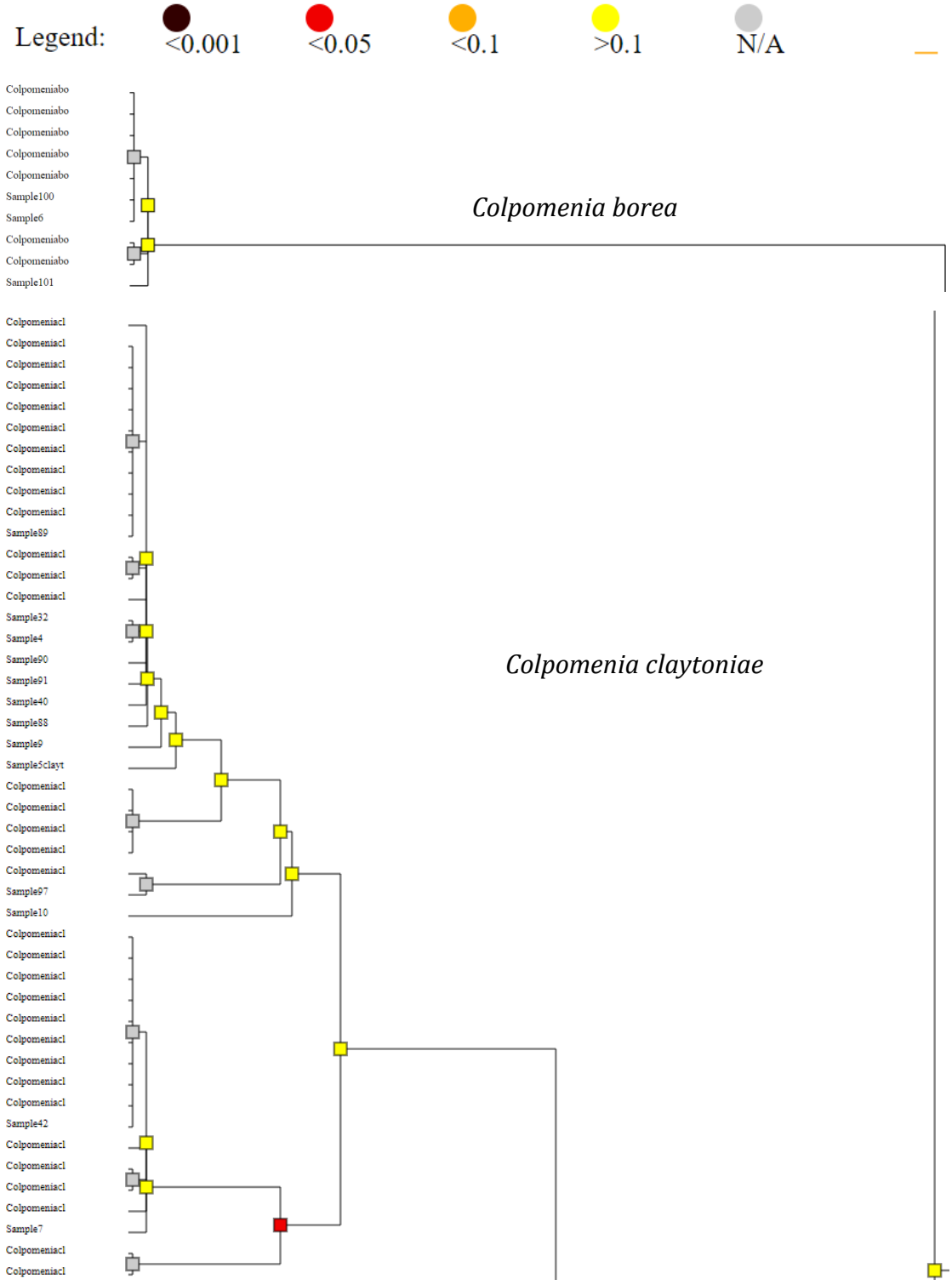

Figure 26a. *C. borea* and *C. claytoniae* ASAP node groupings.

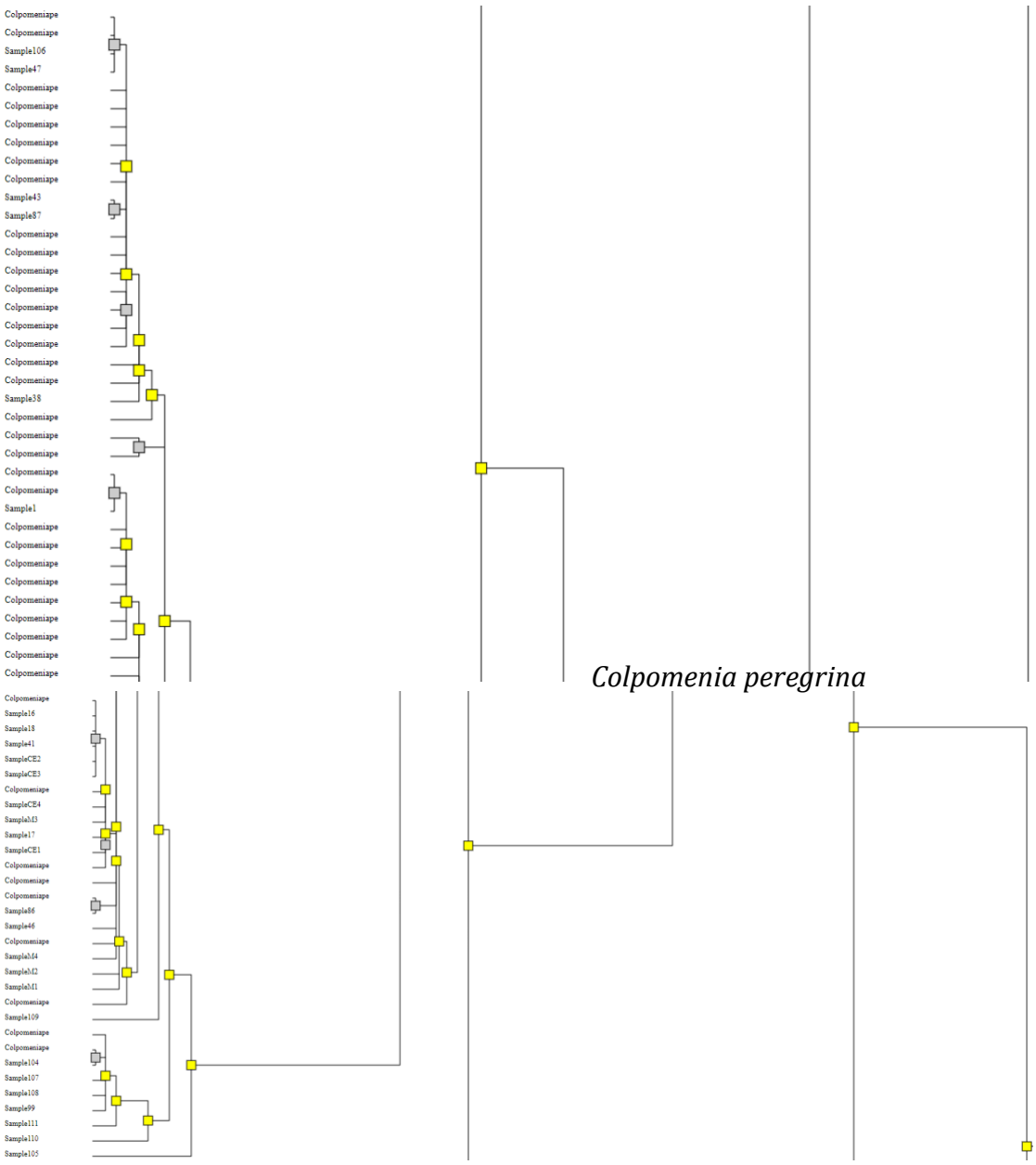

Figure 26b. *C. peregrina* ASAP node groupings.

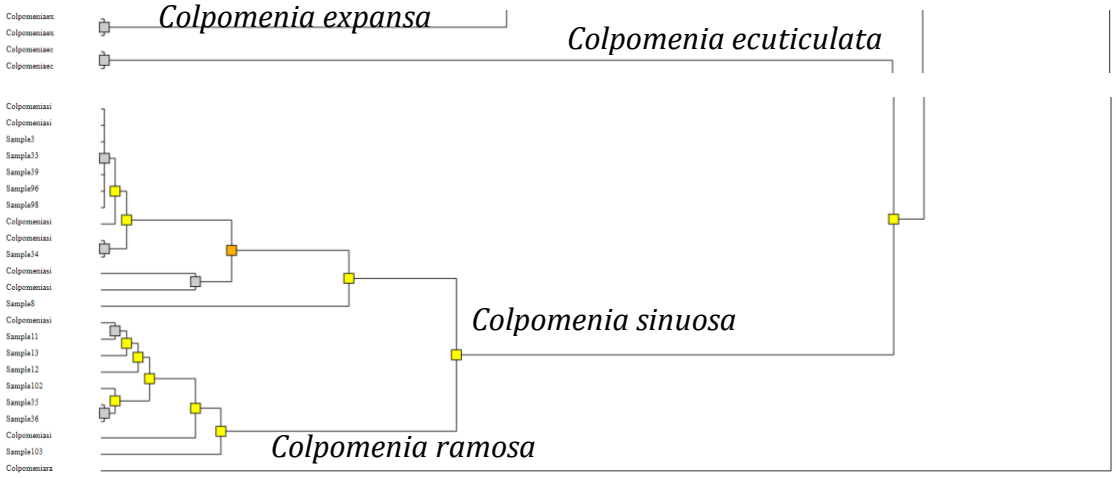

Figure 26c. *C. expansa, C. ecuticulata*, *C. sinuosa*, and *C. ramosa* ASAP node groupings.

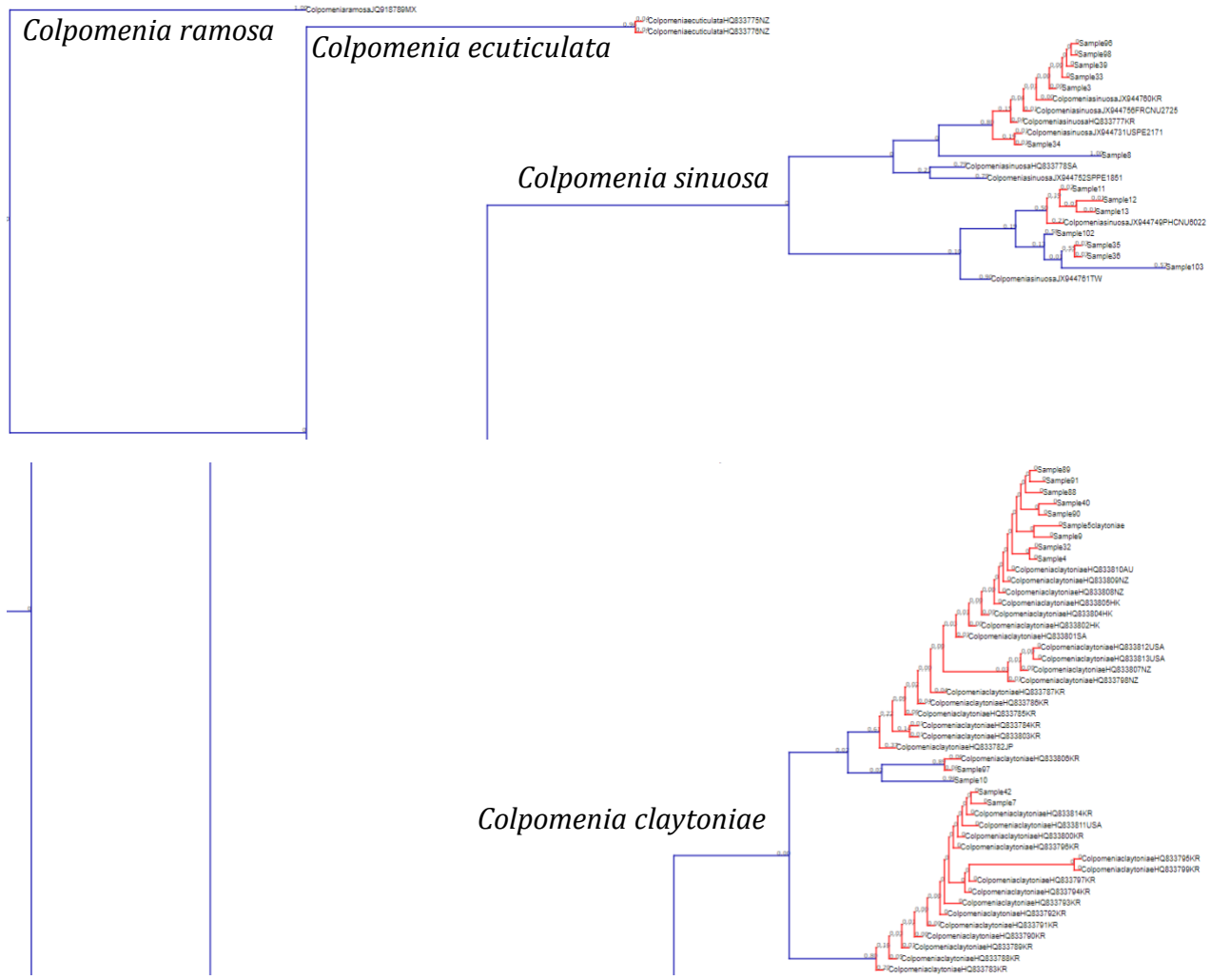

Figure 27a. PTP Tree *C. ramosa*, *C. ecuticulata*, *C. sinuosa*, and *C. claytoniae*.

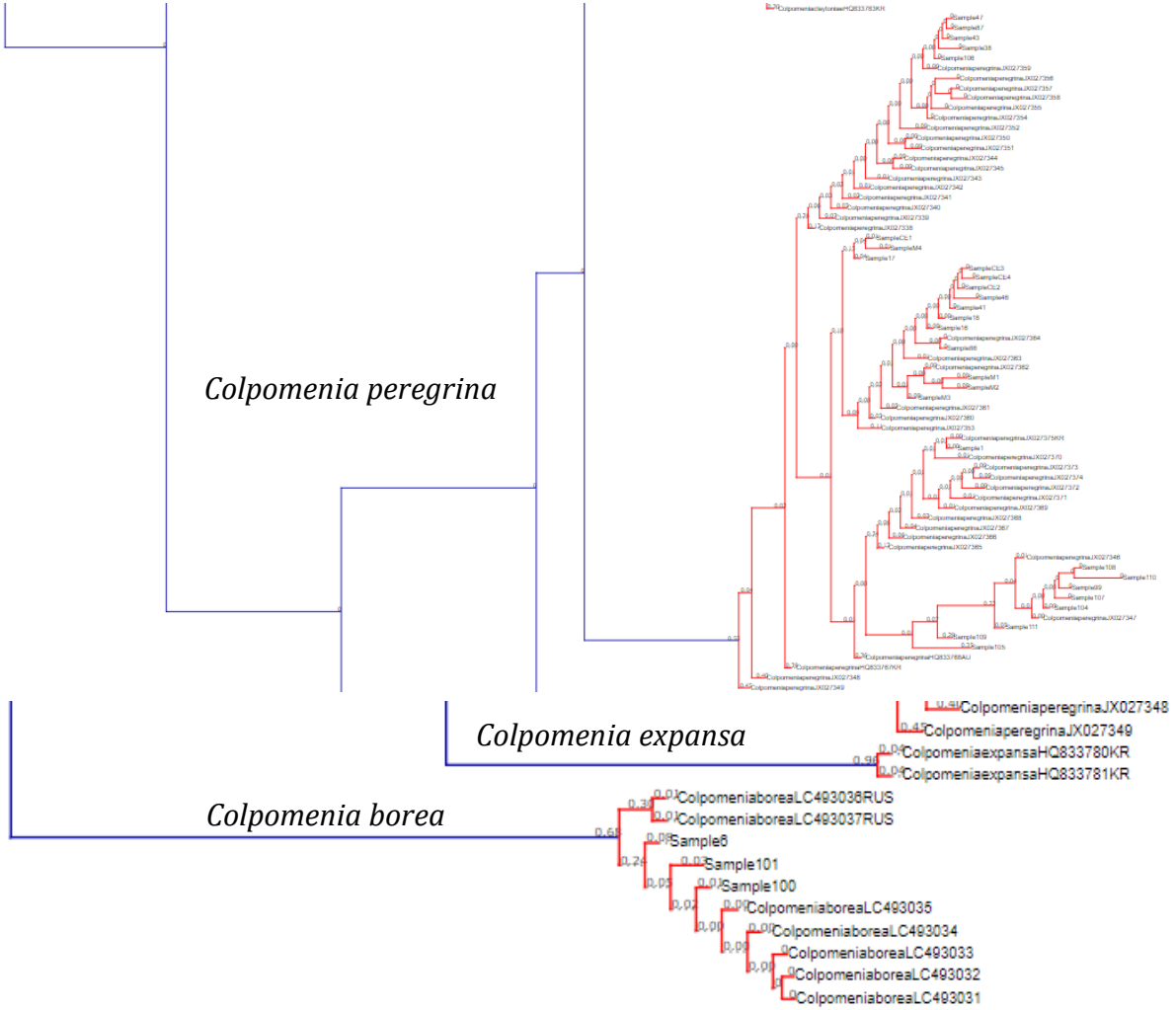

Figure 27b. PTP Tree *C. peregrina*, *C. expansa*, and *C. borea*.

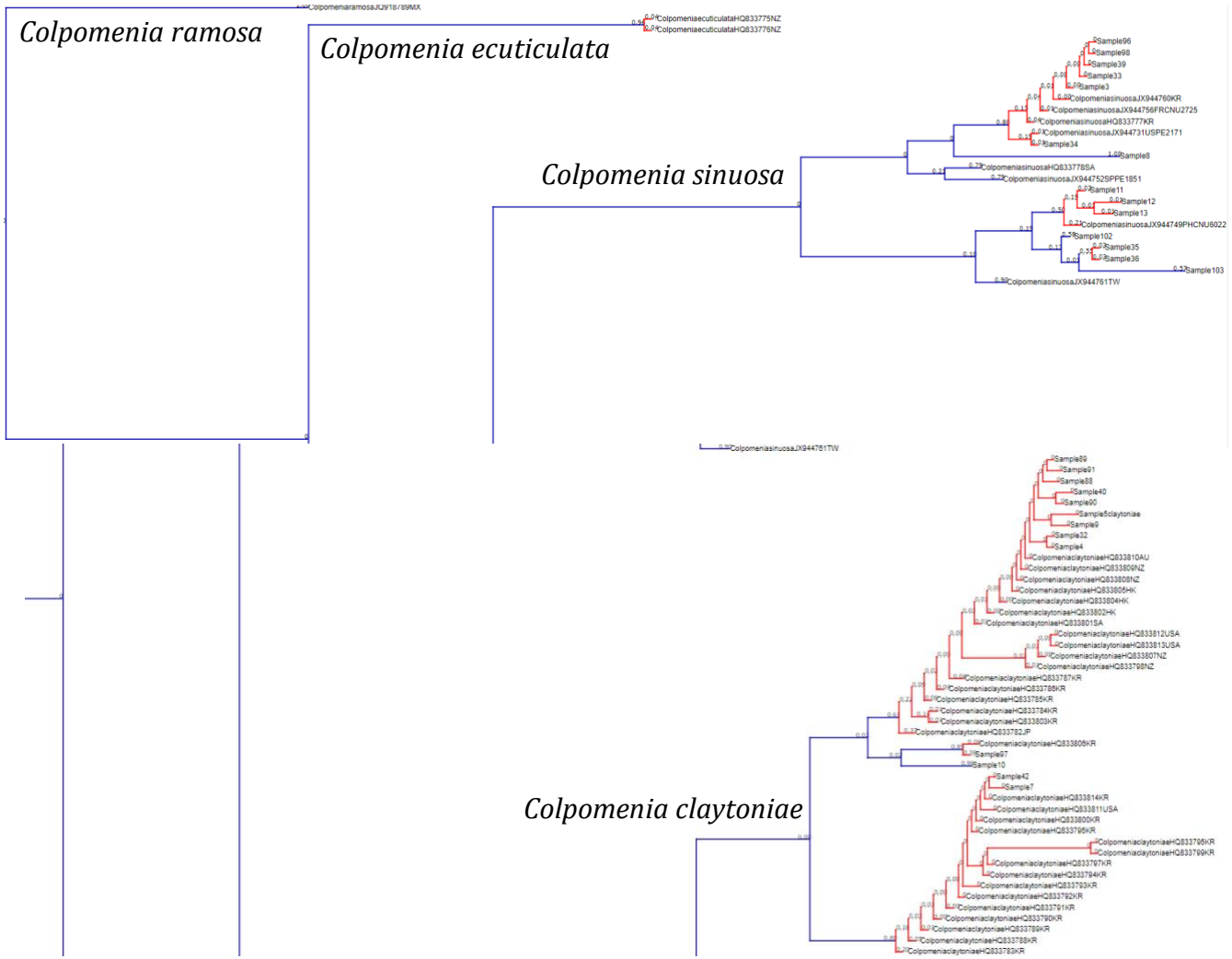

Figure 28a. bPTP Tree *C. ramosa*, *C. ecuticulata*, *C. sinuosa*, and *C. claytoniae*.

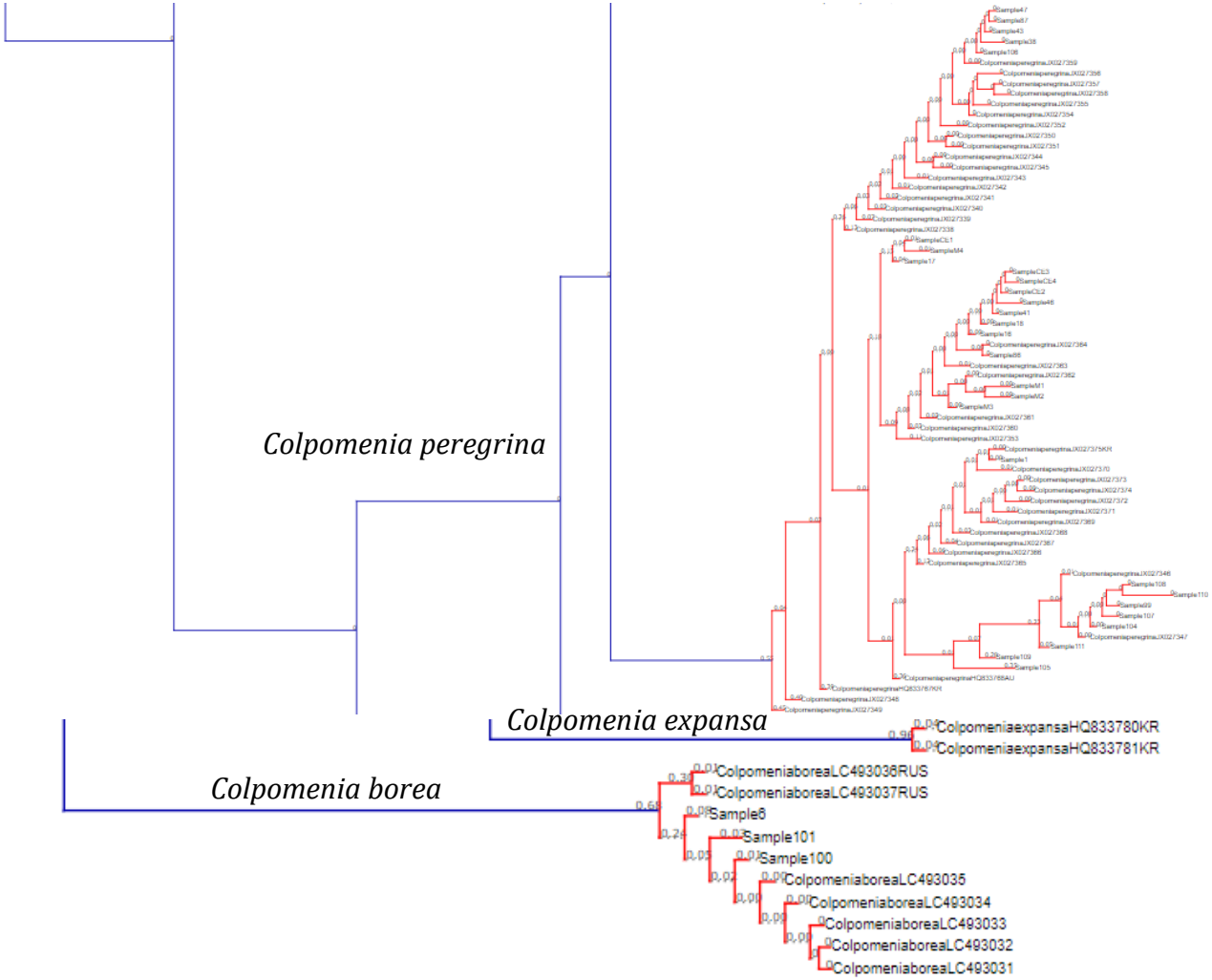

Figure 28b. bPTP Tree *C. peregrina*, *C. expansa*, and *C. borea*.

TABLES

Table 1. Primer lists used in this study.

Genetic Marker	Primer Name	Sequence	References
***cox*3**	F- F49	CATTTAGTNGAYCCWAGYCCTTGGC	Kogame *et al*. 2005
	R- CAR4A	CCCCACCARTAWATNGTNAG	and Boo *et al*. 2010
***rbc*L-spacer-*rbc*S**	F- PRB-F0	ATTAAGAGTTTGTGGTTGGC	Kogame *et al*. 1999 and Yamoto 1997 (for the *rbc*L3F)
	R- PRB-R1A	CCAGAAAGACCTAATTTAGG	
	F- PRB-F2	TTCCAAGGCCCAGCAACAGGT	
	R- PRB-R2	CCTTTAACCATTAAGGGATC	
	F- PRB-F3	TGTAAATGGATGCGTATGTG	
	R- PRB-R3A	GTAATATCTTTCCATAAATCTAA	
	F- *rbc*L3F	CAGGTGCTACAGCTAACCGTGT	
	R- RSPR	AATAAAGGAAGACCCCATAATTCCCA	
***psa*A**	F- 130F	AACWACWACTTGGATTTGGAA	Yoon *et al*. (2002)
	R- 970R	GCYTCTARAATYTCTTTCA	
	F- 870F	GGNGGWYTATGGTTAAGTGA	
	R- 1760R	CCTCTWCCWGGWCCATCRCAWGG	

Table 2. Morphological comparison of *Colpomenia borea* with five globose species of *Colpomenia*.

	Colpomenia borea sp. nov.	*C. sinuosa*	*C. claytoniae*	*C. ecuticulata*	*C. expansa*	*C. peregrina*
Thallus shape	Globose to ovoid, smooth	Globose or vesicle-like, firm, deeply folded	Globose or vesicle-like, irregularly convoluted	Globose or vesicle-like, slightly folded	Globose or vesicle-like, very tiny clumps of hairs on the surface	Globose or vesicle-like
Thallus size (cm)	≤ 5, 150–210	≤ 15, ≤ 500	≤ 30, up to 300	30 to 50 or more, –	≤ 6 –	≤ 10 ≤ 300
Membrane thickness (µm)						
Cortex	pigmented ovoidal, 1–2 layers 6.9–20	pigmented polygonal, 1–6 layers –	pigmented polygonal, 1–2 layers	angular, 1–2 layers	angular, 1–3 layers	polygonal, 1–3 layers

Cell size (width, µm)			–	–	–	–
Medulla	colorless	colorless irregular, 4–	irregular cuboidal,	cuboidal,	cuboidal,	colorless
Cell size	irregularly shaped,	6 layers,	5–6 layers,	3–4 layers,	5–7 layers,	irregular, 3–4
(diameter, µm)	2–3 layers, up to	–	–	–	–	layers,
	125					–
Plurilocular	irregularly ovoid,	punctate associate	extensive	reticulate not	punctate,	extensive,
sporangial sori	extensive,	with a hair pit	cuticle absent	associate with a	–	cuticle absent
	cuticle absent	cuticle absent		hair pit, cuticle		
				absent		
Paraphyses	one-celled	one-celled	one-celled	up to 3 celled	one-celled	one-celled
(ascocysts)						
Size (cross	8–18 × 7.8–22.5	30 long	–	–	–	30 long
section; width ×						
length, µm)						

Habitat	Epiphytic on *Stephanocystis crassipes*, subtidal	usually epilithic on rocks, occasionally epiphytic, lower intertidal to subtidal	epilithic, usually low intertidal	epilithic, subtidal	epilithic, intertidal	usually epiphytic, rarely ephilitic, intertidal to subtidal
Type locality	Akkeshi, Hokkaido, Japan	Cadiz, Spain	Sangjokam, Goseong, South Korea	Takatu Peninsula, North Island, New Zealand	Avalon Bay, Santa Catalina, USA	Morbihan, France
Distribution	Cold waters, Hokkaido, Japan and Russian Far East	Tropical to warm temperate waters worldwide	Cold to tropical waters, Korea, Hong Kong, Oceania, South Africa, USA	Cold to warm waters, Australia and New Zealand	Cold to warm waters, Santa Catalina, USA, Korea	Temperate waters worldwide
References	This study	Clayton 1975, Norris 2010, Lee *et al.* 2013, Song *et al.* 2019	Boo *et al*. 2011, Song *et al.* 2019	Parsons 1982, Womersley 1987	Saunders 1898, Lee 2008	Fletcher 1987, Kogame and Yamagishi 1997, Song *et al.* 2019

Table 3. Morphological characterizations and life history comparison of *Colpomenia* Lineages I–IV in *cox*3 tree.

	Lineage I	**Lineage III**	**Lineage II**				**Lineage IV**
	C. sinuosa	*C. ecuticulata*	*C. borea*	*C. claytoniae*	*C. expansa*	*C. peregrina*	*C. ramosa*
Thallus	Globose or vesicle-like, firm, deeply folded	Sometimes globose or vesicle-like, usually flattened, slightly folded, smooth	Globose to ovoid, smooth	Globose or vesicle-like, irregularly convoluted	Globose or vesicle-like, very tiny clumps of hairs on the surface	Globose or vesicle-like	Adherent clumps, crisp to stiff, highly irregular, subdichotomo -usly to polychotomou -sly
Thallus size (cm)	≤ 15,	30 to 50 or more,	≤ 5,	≤ 30,	≤ 6,	≤ 10,	≤ 8 diameter, ≤ 2 tall

Membrane thickness (μm)	≤ 500	–	150–210	up to 300	–	≤ 300	400–500
Cortex	pigmented polygonal, 1–6 layers	angular, 1–2 layers	pigmented ovoidal, 1–2 layers	pigmented polygonal, 1–2 layers	angular, 1–3 layers	polygonal irregularly arranged, 1–3 layers	small celled
Cell size (width, μm)	–	–	6.9–20	–	–	–	8.5–10
Medulla	colorless irregular, 4–6 layers	cuboidal, 3–4 layers	colorless irregularly shaped, 2–3 layers	irregular cuboidal, 5–6 layers	cuboidal, 5–7 layers	colorless irregular, 3–4 layers	cuboidal, 6–8 layers

Cell size (diameter, µm)	–	–	up to 125	–	–	–	–
Plurilocular sporangial sori	punctate associate with a hair pit, cuticle absent	reticulate not associate with a hair pit, cuticle absent	irregularly ovoid, extensive, cuticle absent	extensive, cuticle absent	punctate	extensive, cuticle absent	cylindrical, uniseriate, 10–12 locules
Paraphyses (ascocysts)	one-celled	up to 3 celled	one-celled	one-celled	one-celled	one-celled	up to 10 celled
Size (cross section; width × length, µm)	30 long	–	8–18 × 7.8–22.5	–	–	30 long	3–7 × 17–28

Habitat	usually epilithic on rocks, occasionally epiphytic, lower intertidal to subtidal	epilithic, subtidal	Epiphytic on Stephanocystis crassipes, subtidal	epilithic, usually low intertidal	epilithic, intertidal	usually epiphytic, rarely epilithic, intertidal to subtidal	entangled with other algae, lower intertidal to subtidal
Life-history	heteromorphic life cycle between globose thalli and	–	heteromorphic life cycle between globose thalli and	–	–	heteromorphic life cycle between globose thalli and	–

Sexual reproduction	pseudodiscoi-d thalli globose thalli were gametophytes and pseudodiscoid thalli were sporophytes forming ascocysts in addition to plurilocular	–	–	–	–	pseudodisc-oid thalli globose thalli were gametophytes and pseudodiscoid thalli were sporophytes forming ascocysts in addition to plurilocular	–
Pseudodiscoid thalli	–	–	pseudodisc-oid thalli	forming ascocysts in addition to plurilocular	–	–	–

	and unilocular sporangia		and unilocular sporangia			and unilocular sporangia	
Plurilocular sporangia on pseudodiscoid thalli shape	ectocarpoid	–	broadly ellipsoid to rectangular	–	–	ectocarpoid	–
Type locality	Cadiz, Spain	Takatu Peninsula, North Island, New Zealand	Akkeshi, Hokkaido, Japan	Sangjokam, Goseong, South Korea	Avalon Bay, Santa Catalina, USA	Morbihan, France	Isla Cedros, Baja California, Mexico

Distribution	Tropical to warm temperate waters worldwide	Cold to warm waters, Australia and New Zealand	Cold waters, Hokkaido, Japan and Russian Far East	Cold to tropical waters, Korea, Hong Kong, Oceania, South Africa, USA	Cold to warm waters, Santa Catalina, USA, Korea	Temperate waters worldwide	Warm waters
References	(Clayton 1975, 1979; Kogame 1997; Kogame & Yamagishi 1997; Toste *et al*. 2003; Norris 2010;	(Parsons 1982; Womersley 1987)	(Dy *et al*. 2023)	(Boo *et al*. 2011; Song *et al*. 2019)	(Saunders 1898; Lee 2008)	(Clayton 1979; Fletcher 1987; Kogame and Yamagishi 1997; Toste *et al*. 2003;	(Taylor 1945, Wynne and Norris 1976, Norris 2010)

Lee *et al*. 2013; Song *et al*. 2019)	Norris 2010; Lee *et al*. 2013; Song *et al*. 2019)

Table 4. Specimens of *Colpomenia borea* examined in this study.

Locality and date	Species (identified as)	Voucher specimen	Coordinates	Habitat	Collected by
Muroran, Hokkaido, Japan (in harbor) 2 April 1935	(*Colpomenia sinuosa*)	SAP023185	42°18'39.729"N, 140°58'3.471"E	On *Stephanocystis crassipes*	T. Muraoka
Muroran, Hokkaido, Japan (in harbor) 2 April 1935	(*Colpomenia sinuosa*)	SAP023186	42°18'39.729"N, 140°58'3.471"E	On *Stephanocystis crassipes*	T. Muraoka
Akkeshi, Hokkaido, Japan 26 and 29 April 1991	(*Colpomenia peregrina*)	SAP059264	43°01'16.9"N, 144°50'12.5"E	On *Stephanocystis crassipes*	K. Kogame
Nemuro, Hokkaido, Japan 28 June 1969	(*Colpomenia peregrina*)	SAP087309	43°21'52.8"N, 145°37'25.3"E	On *Stephanocystis crassipes*	M. Kurogi

Nemuro, Hokkaido, Japan 29 June 1969	(*Colpomenia peregrina*)	SAP087310	43°21'52.8"N, 145°37'25.3"E	On *Stephanocystis crassipes*	M. Kurogi
Nokkamappu, Nemuro, Hokkaido, Japan 31 July 1969	(*Colpomenia peregrina*)	SAP087311	43°23'12.7"N, 145°40'26.2"E	On *Stephanocystis crassipes*	M. Kurogi
Aikkappu, Akkeshi, Hokkaido, Japan 27 June 1934	(*Colpomenia peregrina*)	SAP112234	43°00'51.6"N, 144°49'55.3"E	On *Stephanocystis crassipes*	Y. Yamada and T. Tanaka
Aikkappu, Akkeshi, Hokkaido, Japan 27 June 1936	(*Colpomenia peregrina*)	SAP112235	43°00'51.6"N, 144°49'55.3"E	On *Stephanocystis crassipes*	Y. Yamada and T. Tanaka
Daikoku Island, Akkeshi, Hokkaido, Japan	(*Colpomenia peregrina*)	SAP112236	42°57'23.6"N, 144°52'05.7"E	On *Stephanocystis crassipes*	Y. Yamada and T. Tanaka

27 June 1933					
Akkeshi, Hokkaido, Japan 29 June 2011	*Colpomenia borea*	SAP115466	43°01'16.9"N, 144°50'12.5"E	On *Stephanocystis crassipes*	T. Abe
Akkeshi, Hokkaido, Japan 29 June 2011	*Colpomenia borea*	SAP115467	43°01'16.9"N, 144°50'12.5"E	On *Stephanocystis crassipes*	T. Abe
Akkeshi, Hokkaido, Japan 29 June 2011	*Colpomenia borea*	SAP115468	43°01'16.9"N, 144°50'12.5"E	On *Stephanocystis crassipes*	T. Abe
Akkeshi, Hokkaido, Japan 29 June 2011	*Colpomenia borea*	SAP115469	43°01'16.9"N, 144°50'12.5"E	On *Stephanocystis crassipes*	T. Abe
Akkeshi, Hokkaido, Japan 29 June 2011	*Colpomenia borea*	SAP115470	43°01'16.9"N, 144°50'12.5"E	On *Stephanocystis crassipes*	T. Abe

Akkeshi, Hokkaido, Japan 14 July 2011	*Colpomenia borea*	SAP115471	43°01'16.9"N, 144°50'12.5"E	On *Stephanocystis crassipes*	K. Kogame
Akkeshi, Hokkaido, Japan 14 July 2011	*Colpomenia borea*	SAP115472	43°01'16.9"N, 144°50'12.5"E	On *Stephanocystis crassipes*	K. Kogame
Akkeshi, Hokkaido, Japan 10 June 2013	*Colpomenia borea*	SAP115473[a] (Holotype)	43°01'16.9"N, 144°50'12.5"E	On *Stephanocystis crassipes*	K. Kogame
Akkeshi, Hokkaido, Japan 09 July 2017	*Colpomenia borea*	SAP115474[a]	43°01'16.9"N, 144°50'12.5"E	On *Stephanocystis crassipes*	M. J. C. Dy and M. Hoshino
Magadan, Far East Russia Svetlaya Bay 11 July 2016	*Colpomenia borea*	SAP115475[a]	59°29'30.5"N, 150°42'17.4"E	On *Stephanocystis crassipes*	N. Yotsukura, N. Klochkova, T. Abe, and K. Kogame

Magadan, Far East Russia Svetlaya Bay 15 July 2016	*Colpomenia borea*	SAP115476[a]	59°29'30.5"N, 150°42'17.4"E	On *Stephanocystis crassipes*	N. Yotsukura, N. Klochkova, T. Abe, and K. Kogame
Magadan, Far East Russia Svetlaya Bay 15 July 2016	*Colpomenia borea*	SAP115477[a]	59°29'30.5"N, 150°42'17.4"E	On *Stephanocystis crassipes*	N. Yotsukura, N. Klochkova, T. Abe, and K. Kogame

Table 5. List of downloaded sequences used in this study.

Species and collection details	GenBank accession number		
	***cox*3**	***rbc*L**	***psa*A**
***Chnoospora minima* (K. Hering) Papenfuss**			
Yonagunijima, Okinawa, Japan	KF700277	DQ239768	DQ239773
Polihua Beach, Lanai, Hawaii, U.S.A.; 27 March 2008		AB578987	
Tahai, Easter Island, Chile; 15 March 2016	MG570399		
	MG570401		
***Colpomenia borea* M. J. C. Dy, M. Hoshino, T. Abe, Yotsukura, K.M. Lee, S.M. Boo, N. Klochkova, & Kogame sp. nov.**			
Akkeshi, Hokkaido, Japan; 10 June 2013 (SAP115473)	**LC493031**		
Magdan, Russia; 11 July 2016 (SAP115475)	**LC493036**	**LC493039**	
Magdan, Russia; 15 July 2016 (SAP115476)	**LC493037**		
Akkeshi, Hokkaido, Japan; 09 July 2017 (SAP115474)	**LC493032**	**LC493038**	
Akkeshi, Hokkaido, Japan; 09 July 2017 (SAP115474)	**LC493033**		

Akkeshi, Hokkaido, Japan; 09 July 2017 (SAP115474)	**LC493034**	
Akkeshi, Hokkaido, Japan; 09 July 2017 (SAP115474)	**LC493035**	
***Colpomenia claytoniae* S. M. Boo, K. M. Lee, G. Y. Cho & W. Nelson**		
Coral St. Beach, California, USA; 11 December 1999	HQ833811	
Hupo, Uljin, Korea; 10 April 2001	HQ833783	
Gellibrand Reserve, Melbourne, Australia; 07 August 2001	HQ833810	AY398450
Anin, Gangreung, Korea; 12 January 2002	HQ833787	
Hansuri, Hanrim, Korea; 4 December 2002	HQ833799	
Sangjokam, Goseong, Korea; 9 December 2003	HQ833794	
	HQ833796	
	HQ833797	
Seaforth Road, Capetown, South Africa; 3 February 2004	HQ833801	
Island Bay, Wellington, New Zealand; 30 July 2004	HQ833798	
Sangjokam, Goseong, Korea; 12 January 2005	HQ833784	
	HQ833785	
	HQ833786	

Nakura, Ishigaki, Japan; 6 February 2005	HQ833782	
Hupo, Uljin, Korea; 22 May 2005		KJ418173
Sinsudo, Chujado, Korea; 24 May 2005	HQ833800	
Hupo, Uljin, Korea; 25 May 2005	HQ833814	
Aninjin, Gangreung, Korea; 9 February 2006	HQ833803	
Jumunjin, Gangreung, Korea; 20 February 2006	HQ833791	
	HQ833792	
Sangjokam, Goseong, Korea; 1 March 2006	HQ833795	
Urupukapuka, Bay of Islands, New Zealand; 8 April 2006	HQ833808	
Wilson Bay, Coromandel, New Zealand; 10 September 2006	HQ833807	
Dolsando, Yeosu, Korea; 24 June 2007	HQ833790	
	HQ833788	
	HQ833789	

Lobster Bay, Hong Kong; 23 April 2008	HQ833802	
	HQ833805	
Stephenie Bay, Hong Kong; 23 April 2008	HQ833804	
Shinyang, Seoguipo, Korea; 6 June 2009	HQ833806	
Sacheon, Gangreung, Korea; 25 June 2009	HQ833793	
Urquharts Bay, Whangarei Harbour, New Zealand; 28 September 2009	HQ833809	
SanPedro, California; 9 June 2010	HQ833812	
	HQ833813	
***Colpomenia ecuticulata* M. J. Parsons**		
Horseshoe Bay, Marlborough Sounds, New Zealand; 19 October 2005	HQ833775	
Marsden Point, Whangarei Harbour, New Zealand; 05 November 2009	HQ833776	
***Colpomenia expansa* D. A. Saunders**		
Mukri, Chujado, Korea; 24 May 2009	HQ833781	JQ918816

Damuraemi, Chujado, Korea; 26 May 2009	HQ833780		
***Colpomenia peregrina* Sauvageau**			
Oshoro, Hokkaido, Japan; 15 April 1991		AB022235	
Sacheon, Gangreung, Korea; 23 February 1999	HQ833767		
Monterey, California, USA; 11 December 1999		AY398464	
Jawbone Reserve, Melbourne, Australia; 07 April 2001	HQ833768		
Nakhodka, Vladivostok, Russia; 23 May 2002		AY398461	
Sanjokam, Goseong, Korea; 12 January 2005	JX027375		
Anin, Gangreung, Korea; date unknown			DQ239776
***Colpomenia ramosa* W. R. Taylor**			
Punta La Esmeralda, Baja, Mexico; 02 December 2006	JQ918789		
***Colpomenia sinuosa* (Mertens ex Roth) Derbès & Solier**			
Kasumi, Hyogo Prefecture, Japan; 02 August 1990		AB022234	
Tamarana Beach, Sydney, Australia, 11 Aug. 2001		AY398470	
Guryongpo, Pohang, Korea; 16 Novovember 2002			AY372950
Guryongpo, Pohang, Korea; date unknown		AY623653	
Hanrim, Jejudo, Korea; 4 December 2002		AY398468	

Tonggumi, Ulreungdo, Korea; 27 June 2003	JX944760		
Sail Rock, Kaohsiung, Taiwan, 24 March 2004	JX944761		
Castillo san Cristobal, Gran Canaria, Spain; 25 April 2004		AY875710	
Black Bock, South Africa; 10 August 2005	HQ833778		
Haleiwa Beach Park, Oahu, Hawaii, USA; 18 May 2007		AB578988	
Theoule, Cannes, France; 5 June 2007	JX944756		
Gimoto, Catanduanes, Philippines; 18 April 2009	JX944749		
Sacheon, Gangreung, Korea; 25 June 2009	HQ833777		
Tarifa, Cadiz, Spain; 12 April 2010	JX944752		
Shaw 's Cove, CA, USA; 27 June 2010	JX944731		
Hansuri, Jeju, Korea; date unknown		AY398469	
Safat, Kuwait; unknown date			MT683810
			MT683812

***Dactylosiphon bullosus* (D.A.Saunders) W. J. E. Santiañez, K. M. Lee, S. M. Boo and K. Kogame**

Muroran, Hokkaido, Japan; 15 February 1991		AB022236	
Mukri, Chujado, Korea; 26 April 2005	HQ833770		

Kainga Reef, Burrewarra Point, NSW, Australia; 30 December 2008		GU014704	
Kaikoura, Canterbury, New Zealand; January 2011	JQ918800		
Bamfield, Vancouver Island; unknown date			DQ239774
***Dactylosiphon durvillei* (Bory de Saint-Vincent) W. J. E. Santiañez, K. M. Lee, S. M. Boo & K. Kogame**			
Las Cuevas, Sonora, Mexico; 31 March 2009	JQ918801	JQ918825	
Los Molles, Coquimbo, Chile, 28 October 2011	JQ918812	JQ918823	
***Dactylosiphon wynnei* (K. M. Lee, R. Riosmena-Rodriguez, Kogame & S. M. Boo) Santiañez, K. M. Lee, S. M. Boo & K. Kogame**			
Tsuyazaki, Fukuoka, Japan; 03 May 1989		AB022237	
Hoedong, Jindo, Korea; 09 March 2001		AY398467	
Sangjokam, Goseong, Korea; 21 January 2011	JQ918815		
Hoedong Korea; 12 December 2014	KF700278		
Oura, Japan; 12 December 2014	KF700299		
Nagasaki, Japan; 12 December 2014		KF700327	

		KF700328	
***Encephalophycus tuberculatus* (D.A. Saunders) Santiañez**			
El Sargento, Baja California, Mexico; 11 May 2009	HQ833774	JQ918817	
Latin America; 07 February 2014		KJ418174	
***Hapterophycus canaliculatus* Setchell et N.L. Gardner**			
Oshoro, Hokkaido, Japan; 16 May 1990	MH854517		
San Clemente Island, CA, U.S.A., 17 October 2009	KF700321		
Oshoro, Hokkaido, Japan; 9 May 2011	MH854518		MH854515
***Hydroclathrus clathratus* (C. Agardh) M.A. Howe**			
Sado, Niigata, Japan; 8 July 1990		AB022233	
Tsuyazaki, Fukuoka, Japan; 7 March 1999			AY372951
Sakurajima Island, Kagoshima, Japan; 29 March 2006	MF431998	MF431949	
Tassya, Sado I., Niigata, Japan; 17 August 2015	MF432043		
	MF432042		
***Hydroclathrus rapanuii* Santiañez, Macaya et Kogame**			

Vaihu, Easter Island, Chile; 20 March 2016	MG450660	MG251837	MG450664
	MG450661		
	MG584830		
***Hydroclathrus tilesii* (Endlicher) Santiañez & M. J. Wynne**			
Kahala, Oahu, Hawaii; 14 June 2007	MF432045		
Sumuide, Nago, Okinawa, Japan; 28 April 2013	MF432032		
Innoshima Island, Hiroshima, Japan; 20 April 2015		MF431946	
Azores, Portugal; May 2016	MF432055		
Changwido, Jeju, Korea; date unknown			DQ239778
***Hydroclathrus tenuis* Tseng & Lu**			
Polihua, Lanai, Hawaii; 27 March 2008	MF432047		
Sumuide, Nago, Okinawa, Japan; 28 April 2013	MF431997	MF431950	
Panglao I., Bohol, Philippines; 6 June 2015	MF432039		

Batac, Ilos Cortes, Phillipines; date unknown		DQ239770	DQ239779
***Iyengaria stellata* (Børgesen) Børgesen**			
Knysna, Western Cape, South Africa; 11 December 2012		MT773447	
Nature's Valley, Western Cape, South Africa; 12 December 2012			MT773450
***Manzaea minuta* Santiañez & Kogame**			
Taketomi, Okinawa, Japan; 24 March 2001	MF432114	AB578989	
Senaga, Naha, Okinawa, Japan; 30 March 2009		MF431948	MF431956
***Melanosiphon intestinalis* (De A. Saunders) M. J. Wynne**			
Canada; 14 September 2005		KF293726	
Kruz of Island, Sitka, Alaska; Jul 13, 2006		KM587022	
Shizunai, Hokkaido, Japan; 14 July 2013		LC472401	
***Myelophycus caespitosus* Kjellman**			
Shiriya, Aomori, Japan; 9 August 2014	LC471320		
Kirikiri, Iwate, Japan; 21 April 2015	LC215852		
Hidaka, Wakayama, Japan; 7 April 2016	LC471330		
Shibushi, Kagoshima, Japan; 14 May 2017		LC472386	

Woongdo, Taean, Korea; date unknown		AY095319	
***Myelophycus cavus* Tanaka et Chihara**			
Woongdo, Taean, Korea; 15 June 2000	KF700315	AY095319	DQ239781
Kumage, Yamaguchi, Japan; 17 April 2009	LC471318		
***Myelophycus simplex* (Harvey) Papenfuss**			
Kurohae Beach, Chiba, Japan; 31 July 2004	KF700316		
Daesado, Wando, Korea; date unknown			AY372952
***Petalonia binghamiae* (J.Agardh) K.L. Vinogradova**			
Puraengi, Chujado, Korea; 23 May 2004	KF700317		
Kasumi, Hyogo, Japan; 3 June 1991		AB022244	
Munseom, Jeju, Korea; date unknown			DQ239782
***Petalonia fascia* (O. F. Müller) O. Kuntze**			
Ohma, Aomori Prefecture, Japan; 02 February 1990		AB022243	
IledeBatz, Roscoff, France; 5 April 2000			AY372953
Munseom, Jejudo, Korea; 1 April 2011	HQ833766		
***Petalonia tatewakii* Kogame *et* A. Kurihara**			
Halawa, Molokai, Hawaii, USA; 12 December 2009		AB579008	

***Petalonia tenella* (Kogame) Santiañez**			
Muroran, Hokkaido, Japan; 22 January 1989		AB022241	
***Petalonia tenuis* Matsumoto et Shimada**			
Isshiki, Hayama, Kanagawa, Japan; 30 December 2010		AB860192	
***Planosiphon complanatus* (Rosenvinge) McDevit et G.W. Saunders**			
Bay of Fundy, NB, Canada; 22 March 2007		KF281389	
***Planosiphon gracilis* (Kogame) McDevit & G. W. Saunders**			
Hado, Jeju, Korea; 22 March 2000	KF700323		DQ239786
Misaki, Osaka, Japan; 4 January 2017		LC472406	
***Planosiphon nakamurae* M.Hoshino, M.E.Croce, Hanyuda & Kogame**			
Samani, Hokkaido, Japan; 19 May 1991		LC490561	
Oregon, USA; 20 January 2015	LC483670		
Ooiso, Hyogo, Japan; 25 March 2016	LC483675		

***Planosiphon zosterifolius* (Reinke) McDevit & G.W.Saunders**			
Onyangri, Uljin, Korea; 12 January 2002	KF700318		DQ239783
Oga, Akita, Japan; 18 February 2017	LC471355	LC472407	
***Pseudochnoospora implexa* (J. Agardh) Santiañez, G.Y. Cho et Kogame comb. nov.**			
Sesoko, Okinawa, Japan; 8 March 1990		AB022231	DQ2397772
Port-Boisé, New Caledonia; date unknown	GQ368273		
***Pylaiella littoralis* (Linnaeus) Kjellman**			
Dinard, Brittany Coast, France; 21 April 2010		KM057702	
***Rosenvingea australis* Huisman, G.H. Boo *et* S.M. Boo**			
Cabilao Island, Bohol, Philippines; 11 May 2014			MN224305
Cape Peron, Perth, Western Australia, Australia; 26	MG544889		MH170371
February 2015	MG544892		
***Rosenvingea intricata* (J. Agardh) Børgesen**			
Gushikawa, Okinawa, Japan; 21 January 1997		AB022232	
Playa La Concha, La Paz, Mexico; 31 March 2009	KM587011		

Isla Canal de Afuera, Veraguas, Panama; 14 January 2011			KM587024
			DQ239784
Cam Ranh Bay, Nha Trang, Vietnam; 29 March 2011			KM587023
***Rosenvingea orientalis* (J. Agardh) Børgesen**			
Cam Ranh Bay, Nha Trang, Vietnam; 9 April 2011	KM587019		KM587025
Manjagaw, Surigao, Philippines; date unknown	MG450659	MG251836	MG450665
***Scytosiphon dotyi* M.J.Wynne**			
Monterey Bay, CA, USA; 11 December 1999	KF700322		DQ239785
***Scytosiphon lomentaria* (Lyngbye) Link**			
Seongsan, Jeju, Korea; 22 March 2000			AY372954
Kannonzaki, Kanagawa, Japan; 2 April 2000		LC517918	
Sormsangi, Chujado, Korea; 23 May 2005	HQ833765		
Kirikiri, Iwate, Japan; 21 April 2015	LC215852		
***Tronoella ryukyuana* Santiañez *et* K. Kogame**			
Odo, Itoman, Okinawa, Japan; 28 March 2009	MF432012	MF431947	MF431952
Adenocystis utricularis (Bory) Skottsberg			
Barton, MaxwellBay, Antarctica; 25 January 2000			AY372939

***Chordaria flagelliformis* (O.F. Müller) C. Agardh**			
Sashirui, Shiretoko, Hokkaido, Japan		AB066086	
Avacha Bay, Kamchatka, Russia; 24 July 1998		AY095324	AY372941
Schleimünde, Germany; date unknown	JF796553		
***Ectocarpus siliculosus* (Dillwyn) Lyngbye**			
San Juan de Marcona, Peru; 1988	FP885846		
Hoedong, Jindo, Korea, 9 March 2001		AY372978	AY372949

Table 6. Information on all samples used in this study.

Sample codes	Collection dates			Localities			Species name	Habitat	Collected by	Voucher specimen
1	2008	June	19	Shishiiwa	Shiretoko	Japan	*Colpomenia peregrina*	-	-	
2	2011	July	14	Akkeshi	Hokkaido	Japan	*Colpomenia borea*	-	K. Kogame	
3	2012	June	25	Tsuyazaki	Fukuoka	Japan	*Colpomenia sinuosa*	-	D. Kinoshita	SAP115652
4	2012	June	25	Tsuyazaki	Fukuoka	Japan	*Colpomenia claytoniae*	-	D. Kinoshita	SAP115652
5	2013	April	27	Ooura	Okinawa	Japan	*Colpomenia peregrina*	-	-	SAP115653
6	2013	June	10	Barasan	Hokkaido	Japan	*Colpomenia borea*	Drifted	-	
7	2016	March	9	Faro		Portugal	*Colpomenia claytoniae*	-	M. Hoshino	
8	2016	March	11	Pria De Faro		Portugal	*Colpomenia sinuosa*	-	M. Hoshino	
9	2016	March	11	Pria De Faro		Portugal	*Colpomenia claytoniae*	-	M. Hoshino	
10	2016	July	9	Coral Bay Western		Australia	*Colpomenia claytoniae*	-	J. Huisman	
11	2016	July	11	Coral Bay Western		Australia	*Colpomenia sinuosa*	-	J. Huisman	
12	2016	July	11	Coral Bay Western		Australia	*Colpomenia sinuosa*	-	J. Huisman	

				Coral Bay						
13	2016	July	11	Western		Australia	*Colpomenia sinuosa*	-	J. Huisman	
16	2017	April	28	Oshoro, Otaru	Hokkaido	Japan	*Colpomenia peregrina*	-	M. Hoshino	SAP115654
17	2017	April	28	Oshoro, Otaru	Hokkaido	Japan	*Colpomenia peregrina*	-	M. Hoshino	SAP115654
18	2017	April	28	Oshoro, Otaru	Hokkaido	Japan	*Colpomenia peregrina*	-	M. Hoshino	SAP115654
33	2006	March	29	Sakurajima	Kyushu	Japan	*Colpomenia sinuosa*	-	K. Kogame	SAP115655
34	2006	March	29	Sakurajima	Kyushu	Japan	*Colpomenia sinuosa*	-	K. Kogame	SAP115655
				Haemida						
35	2008	March	12	Iriomote-jima	Okinawa	Japan	*Colpomenia sinuosa*	-	K. Kogame	
				Haemida						
36	2008	March	12	Iriomote-jima	Okinawa	Japan	*Colpomenia sinuosa*	-	K. Kogame	
								on		
				Moroiso				*Sargassum*		SAP115656
37	2008	March	24	(Shoiso Bay)	Kanagawa	Japan	*Colpomenia peregrina*	*hemiphyllum*	K. Kogame	
								on		
				Moroiso				*Sargassum*		
38	2008	March	24	(Shoiso Bay)	Kanagawa	Japan	*Colpomenia peregrina*	*hemiphyllum*	K. Kogame	SAP115656
				Moroiso						
39	2008	March	24	(Shoiso Bay)	Kanagawa	Japan	*Colpomenia sinuosa*	-	K. Kogame	

				Moroiso						
40	2008	March	24	(Shoiso Bay)	Kanagawa	Japan	*Colpomenia claytoniae*	-	K. Kogame	SAP115657
								Attached on		
41	2018	February	28	Oshoro	Hokkaido	Japan	*Colpomenia peregrina*	*Sargassum*	M. Hoshino	
				Nabetahama,				Attached on		
42	2018	February	20	Shimoda	Shizuoka	Japan	*Colpomenia claytoniae*	rock	M. Hoshino	
				Nabetahama,				Attached on		
43	2018	February	20	Shimoda	Shizuoka	Japan	*Colpomenia peregrina*	*Sargassum*	M. Hoshino	
46	2018	March	21	Oshoro, Otaru	Hokkaido	Japan	*Colpomenia peregrina*	-	M. Hoshino	
47	2018	May	17	Muroran	Hokkaido	Japan	*Colpomenia peregrina*	Floating	M. J. C. Dy	
				Nabetahama,						
87	2018	February	20	Shimoda	Shizuoka	Japan	*Colpomenia peregrina*	-		
88	2018	March	29	Inoshiri	Kochi	Japan	*Colpomenia claytoniae*	-	M. Hoshino	
89	2018	March	29	Inoshiri	Kochi	Japan	*Colpomenia claytoniae*	-	M. Hoshino	
90	2018	March	29	Inoshiri	Kochi	Japan	*Colpomenia claytoniae*	-	M. Hoshino	
91	2018	March	29	Inoshiri	Kochi	Japan	*Colpomenia peregrina*	-	M. Hoshino	SAP0000281
96	2012	April	22	Ejiri, Miyazu	Kyoto	Japan	*Colpomenia sinuosa*	-	D. Kinoshita	SAP115658
				Amarube,						
97	2012	April	23	Kasumiku	Hyogo	Japan	*Colpomenia claytoniae*	-	D. Kinoshita	SAP115659

98	2004	February	25	Kozakai, Himi	Toyama	Japan	*Colpomenia sinuosa*	-	K. Kogame	SAP115660
99	2005	May	10	Hakodate	Hokkaido	Japan	*Colpomenia peregrina*	-	K. Kogame	SAP115661
100	2011	June	29	Akkeshi	Hokkaido	Japan	*Colpomenia borea*	-	T. Abe	
101	2011	June	29	Akkeshi	Hokkaido	Japan	*Colpomenia borea*	-	T. Abe	
102	2009	March	30	Senaga, Tomigusuku	Okinawa	Japan	*Colpomenia sinuosa*	-	K. Kogame	SAP115662
103	2009	March	30	Senaga, Tomigusuku	Okinawa	Japan	*Colpomenia sinuosa*	-	K. Kogame	SAP115662
104	2011	July	17	Kikonai	Hokkaido	Japan	*Colpomenia peregrina*	-	K. Kogame	SAP115663
105	2011	July	17	Kikonai	Hokkaido	Japan	*Colpomenia peregrina*	-	K. Kogame	SAP115663
106	2023	June	4	Tachimachi Cape, Hakodate	Hokkaido	Japan	*Colpomenia peregrina*	-	K. Kogame	SAP115664
107	2023	June	4	Tachimachi Cape, Hakodate	Hokkaido	Japan	*Colpomenia peregrina*	-	K. Kogame	SAP115664
108	2023	June	4	Tachimachi Cape, Hakodate	Hokkaido	Japan	*Colpomenia peregrina*	-	K. Kogame	SAP115664

				Shinori						
109	2023	June	4	Hakodate	Hokkaido	Japan	*Colpomenia peregrina*	-	K. Kogame	SAP115665
				Shinori						
110	2023	June	4	Hakodate	Hokkaido	Japan	*Colpomenia peregrina*	-	K. Kogame	SAP115665
				Shinori						
111	2023	June	4	Hakodate	Hokkaido	Japan	*Colpomenia peregrina*	-	K. Kogame	SAP115665
M1	2021	June	26	Muroran	Hokkaido	Japan	*Colpomenia peregrina*	Punctate	K. Kogame	SAP115666
M2	2021	June	26	Muroran	Hokkaido	Japan	*Colpomenia peregrina*	Punctate	K. Kogame	SAP115666
M3	2021	June	26	Muroran	Hokkaido	Japan	*Colpomenia peregrina*	Punctate	K. Kogame	SAP115666
M4	2021	June	26	Muroran	Hokkaido	Japan	*Colpomenia peregrina*	Punctate	K. Kogame	SAP115666
				Kokunoshima,				Cultured		
CE1	2021	March	8	Takehara	Hiroshima	Japan	*Colpomenia peregrina*	Thalli	M. Hoshino	SAP0000316
				Kokunoshima,				Cultured		
CE2	2021	March	8	Takehara	Hiroshima	Japan	*Colpomenia peregrina*	Thalli	M. Hoshino	SAP0000316
				Kokunoshima,				Cultured		
CE3	2021	March	9	Takehara	Hiroshima	Japan	*Colpomenia peregrina*	Thalli	M. Hoshino	SAP0000321
				Kokunoshima,				Cultured		
CE4	2021	March	9	Takehara	Hiroshima	Japan	*Colpomenia peregrina*	Thalli	M. Hoshino	SAP0000322

Curriculum Vitae

Michael Jacob C. Dy was born on the 2nd of July 1995 in Meycauayan, Bulacan, Philippines. He graduated as a valedictorian and obtained his high school diploma from Messiah School Foundation Inc. in 2011. After graduating from high school, he enrolled at Far Eastern University – Manila campus studying biology. During his last year and a half at the university, he worked as an intern at the University of the Philippines – in the molecular laboratory of the Marine Science Institute under the advisory of Dr. Richard V. Dumilag and with the consent of Dr. Arturo O. Lluisma, and at the Institute of Biology, Herbarium under the supervision of Dr. Sandra Yap. Jake worked on the red algal Rhodophyta as part of their undergraduate thesis. They made interesting discoveries during that time and Jake wants to continue the study of algae. He finished his Bachelor of Science in Biology degree in 2015. Not so long, he was admitted as a master's course student, under the guidance of Dr. Kazuhiro Kogame, in April 2017 and moved to Sapporo, Hokkaido. Jake's research focused on the phylogeny and taxonomy of the genus *Colpomenia*. Upon obtaining his Master of Science in Natural History degree in 2019, Jake continued to the PhD program. His original PhD project was the red algal Delesseriaceae family. Since he is a self-supported student, he worked relentlessly at the same time for more than five years at two English cram school companies as a part-time English teacher to sustain his daily expenses. However, when the COVID-19 pandemic began, everything stopped and left him burned out. Luckily, he found a scholarship from the Sapporo Catholic Church wherein this scholarship covered his tuition fees and apartment rent each month. Jake is forever thankful for the kindness and generosity of the Sapporo Catholic Church Community that made this possible. Without this scholarship, he wouldn't be able to continue his PhD journey. Slowly recovering from the pandemic, he and his supervisor changed his PhD project back to phylogeny, taxonomy, and molecular study of the brown algal genus *Colpomenia* (Scytosiphonaceae).

List of Publications

Dy, M.J.C., Hoshino, M., Abe, T., Yotsukura, N., Klochkova, N., Lee, K.M., Boo, S.M., & Kogame, K. (2023). *Colpomenia borea* sp. nov. (Scytosiphonaceae, Phaeophyceae) from Japan and Far East Russia. *Phycological Research* 71: 81–89.