

## Research Article

Algae 2018, 33(3): 231-241

<https://doi.org/10.4490/algae.2018.33.8.20>

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# Genetic discontinuity of *Digenea* (Rhodomelaceae, Rhodophyta) from Mexico supports recognition of two new species, *D. mexicana* and *D. rafaелиi*

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Genetic continuity of *Digenea simplex* outside the Atlantic waters remains less studied, despite its long exposure to oriental folk medicine and wide distribution in tropical waters. Mitochondrial COI-5P and plastid *rbcL* sequences and morphology were investigated for plants from Mexico and additional specimens from Japan. Two new species, *Digenea mexicana* and *Digenea rafaелиi*, are described for plants that would previously have been recognized as *D. simplex* in Mexico. *D. mexicana* grows to 10.5 cm tall and has cylindrical axes, irregular or di-, trichotomous branches, simple to branched determinate branchlets with eight pericentral cells, and tetrasporangia on inflated upper parts of determinate branchlets. It occurs intertidally in Quintana Roo, Yucatan Peninsula. *D. rafaелиi* grows to 5 cm tall and has cylindrical axes, irregular or di-, trichotomous branches, and simple determinate branchlets with ten pericentral cells. It occurs intertidally in the Gulf of California. Both COI-5P and *rbcL* sequences revealed the genetic discontinuity between *D. mexicana* and *D. rafaелиi*.

**Key Words:** COI-5P; Gulf of California; medicinal seaweeds; morphology; phylogeny; *rbcL*; species complex; Yucatan Peninsula

## INTRODUCTION

*Digenea* C. Agardh is a rhodomelacean red algal genus that until recently was regarded as monospecific, including only *Digenea simplex* (Wulfen) C. Agardh (Agardh 1822). *D. simplex* is widely known as one of the most efficient anthelmintic treatments in oriental folk medicine (Tseng 1983, Trono 1997, Ebadi 2006). The species is also of interest as a source of polysaccharides inhibiting inflammatory and nociceptive responses and the

production of carrageenan (Takano et al. 2003, Pereira et al. 2014). Since its first recognition in the Adriatic Sea, *D. simplex* has been recorded from numerous locations and is now regarded as having a pantropical distribution in the northern and southern hemisphere (Pakker et al. 1996, Silva et al. 1996, Orfanidis and Breeman 1999, Guiry and Guiry 2018).

Agardh (1822) established *Digenea* to accommodate



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Received July 8, 2018, Accepted August 20, 2018

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a species previously known as *Conferva simplex* Wulfen. The genus is characterized by terete, polysiphonous axes, with branches that are densely surrounded by short, stiff, simple filamentous branchlets (Agardh 1822, Kylin 1956, Norris 2014). A second species, *Digenea subarticulata* Simons, was described for plants from KwaZulu-Natal, South Africa (Simons 1970), however, De Clerck et al. (2005) noted that the species likely belongs to the genus *Bryocladia* F. Schmitz. Recently, *Digenea arenahauriens* C. W. Schneider, Hamzeh & G. W. Saunders from Bermuda and *D. cymatophila* (R. E. Norris) Díaz-Tapia & Maggs from Hawaii have been added in the genus (Díaz-Tapia et al. 2017, Schneider et al. 2018).

The apparent pantropical distribution of *D. simplex* has been the focus of biogeographical and taxonomic studies. For example, Pakker et al. (1996) found that Pacific isolates of *D. simplex* survive a broader range of temperatures than Atlantic populations, which may be correlated to local temperature extremes. Orfanidis and Breeman (1999) reported that ecological variation in *D. simplex* was related to the lower tolerance limits of temperature, indicating that the pantropical distribution may be related to evolutionary pressures. Based on DNA sequences and morphology, Schneider et al. (2018) described *D. arenahauriens* for plants from Bermuda that were previously known as *D. simplex*. However, there have been no studies on plants outside the Atlantic, where *D. simplex* is reported to commonly occur (e.g., Yoshida 1998, Norris 2014).

Plants referable to *D. simplex* occur abundantly in the intertidal to subtidal zones of the Yucatan Peninsula and the Gulf of California, Mexico (Dawson 1963, Dreckmann and Senties 1994, Norris 2014). Quintana Roo on the east of the Yucatan Peninsula is an adjacent rocky coast to the Caribbean Sea, comprising mostly sandy bottom, subtidal rocky platforms exposed to the swell, and rocky-sand substrata in reef lagoons. Compared to many studies on the Gulf of California (e.g., Norris 2010, 2014), little is known about marine algae from Quintana Roo because many coastal areas remain unexplored or no descriptions have been made using recent molecular tools. We focused on the genetic discontinuity between *D. simplex* specimens from the Yucatan Peninsula and the Gulf of California. We studied morphology and analyzed mitochondrial COI-5P and plastid *rbcL* sequences, as commonly used in taxonomic studies of red algae (e.g., Boo et al. 2016a, 2016b, Schneider et al. 2018). On the basis of morphology and two genes sequences, we propose two new species of *Digenea*, one from Yucatan Peninsula and the other from the Gulf of California.

## MATERIALS AND METHODS

Samples of *Digenea* were collected from low intertidal zones of the Yucatan Peninsula and the Gulf of California, Mexico, and additional samples from Japan were included (Supplementary Table S1). Vouchers were mounted on herbarium sheets and subsamples were dehydrated in silica gel for molecular analysis. Herbarium specimens in the Herbario Nacional de México (MEXU) of Universidad Nacional Autónoma de México were examined on loan. Vegetative and reproductive structures were observed under a microscope. For anatomical observations, plants were sectioned using razor blades and were stained with 1% aqueous aniline blue. Photographs were taken with a DP-71 camera (Olympus, Tokyo, Japan) mounted on a BX-51 microscope (Olympus). Types and voucher specimens are housed at the Herbarium of the Department of Biology, Chungnam National University, Daejeon, Korea (CNUK) and MEXU of the Universidad Nacional Autónoma de México. Abbreviations of herbaria follow the online Index Herbariorum (Thiers 2018).

DNA extraction, polymerase chain reaction amplification, and sequencing were performed as described in Boo et al. (2013). The primers used for amplifying and sequencing were *rbcL*JNF1-*rbcL*JNR1 and R753-RrbcS start for *rbcL* (Freshwater and Rueness 1994, Kang and Kim 2013), and GWSFn-GazRx for COI-5P (Saunders and Moore 2013). Sequences of COI-5P and *rbcL* generated in this study were deposited in GenBank (Benson et al. 2018). A total of 17 COI-5P sequences (664 bp) were aligned, including sequences of other species of *Digenea* previously published in GenBank. Seventeen *rbcL* sequences (1,220 bp) were aligned including sequences of other species of *Digenea* previously published in GenBank and three outgroup taxa, *Alsidium corallinum* C. Agardh, *Bryothamnion seafortii* (Turner) Kützing, and *B. triquetrum* (S. G. Gmelin) M. Howe (Supplementary Table S1). However, we used phylogeny of COI-5P without outgroups because branch lengths of ingroups were too short to recognize each species.

Phylogenies of COI-5P and *rbcL* datasets were inferred using maximum likelihood (ML) and Bayesian inference (BI). The ML analyses were performed using the Pthreads version of RAxML v8.0.X (Stamatakis 2014) set as follows: a rapid bootstrap analysis and search for the best-scoring ML tree in one single program run with 1,000 bootstrap replicates under GTRGAMMA model. BI was performed for individual datasets with MrBayes v.3.2.1 (Ronquist et al. 2012) using the Metropolis-coupled Markov Chain Monte Carlo (MC3) with the GTR + G + I model. For each

matrix, four million generations of two independent runs were performed with four chains and sampling trees every 100 generations. The burn-in period was identified graphically by tracking the likelihoods at each generation to determine whether they reached a plateau. Twenty-five percent of saved trees were removed, and the remaining trees were used to infer Bayesian posterior probabilities (BPP).

## RESULTS

### *Digenea mexicana* G. H. Boo & D. Robledo sp. nov. (Fig. 1A-J)

**Description.** Plants bushy, up to 10.5 cm high, tough and cartilaginous (Fig. 1A-C); axes sometimes denuded in lower portions, attached by a discoid holdfast which often spreads and may possibly coalesce with other holdfasts; axes terete, irregularly, widely dichotomously or rarely trichotomously branched (Fig. 1B & C); axes and indeterminate branches usually densely (or occasionally sparsely) clothed with wiry, stiff, simple or branched, determinate branchlets of 6.5 mm in length and 90-135  $\mu\text{m}$  in width (Fig. 1F); branchlets polysiphonous, with axial cells 20-30  $\mu\text{m}$  in diameter (Fig. 1D), 8 or rarely 9 pericentral cells surrounded by about 20 cortical cells in one layer (Fig. 1G & H); surface cortical cells oblong, irregularly or longitudinally arranged, in packets of 2-4 cells (Fig. 1E); apices with short vegetative trichoblasts remaining during development; tetrasporangia formed on swollen determinate branchlets, tetrahedrally divided, ellipsoidal to globose, 40-70  $\mu\text{m}$  in diameter (Fig. 1I & J); sexual reproductive structures not found.

**Holotype.** CNU080663 deposited in CNUK, Herbarium of Chungnam National University, Daejeon, Korea; a tetrasporophyte specimen; collected at Playa Xcalacoco (20°39'42.84" N, 87°01'56.78" W), Quintana Roo, Mexico on Apr 19, 2018 by Daniel Robledo.

**Isotypes.** CNU080664, CNU080665, CNU080666, and CNU080668 in CNUK, Korea.

**Additional herbarium specimens observed.** MEXU 2310 (as *D. simplex* determined by José Luis Godínez Ortega; Morro de la Mancha, Veracruz; Nov 8, 2013), MEXU 2326 (as *D. simplex* determined by José Luis Godínez Ortega; Morro de la Mancha; May 18, 2013), MEXU 2357 (as *D. simplex* determined by José Luis Godínez Ortega; Morro de la Mancha; Oct 20, 2011), MEXU 2371 (as *D. simplex* determined by José Luis Godínez Ortega; Mandinga, Veracruz; Apr 7, year unknown), CNU080681

(Playa Xcalacoco, Quintana Roo, Mexico; Jun 16, 2018), CNU080682 (Playa Xcalacoco; Jun 16, 2018), CNU080683 (Playa Xcalacoco; Jun 16, 2018), CNU080684 (Playa Xcalacoco; Jun 16, 2018).

**Etymology.** The specific epithet refers to Mexico, where the plants were collected.

**Distribution and habitat.** Presently confirmed in Playa Xcalacoco, Quintana Roo, Yucatan Peninsula and likely extended to the Gulf of Mexico. *D. mexicana* populations were found growing on intertidal rock, calcareous corallines and reef formations, which are often covered by sands. They formed patches or mats of 1-2 m<sup>2</sup> in area (Fig. 1A).

**DNA sequences of type specimens.** For holotype, MH514874 (COI-5P) and MH514862 (*rbcL*); for isotypes, MH514875-MH514878 (COI-5P) and MH514863-MH514866 (*rbcL*).

### *Digenea rafaellii* G. H. Boo, G. Andrade-Sorcía & S. M. Boo sp. nov. (Fig. 2A-F)

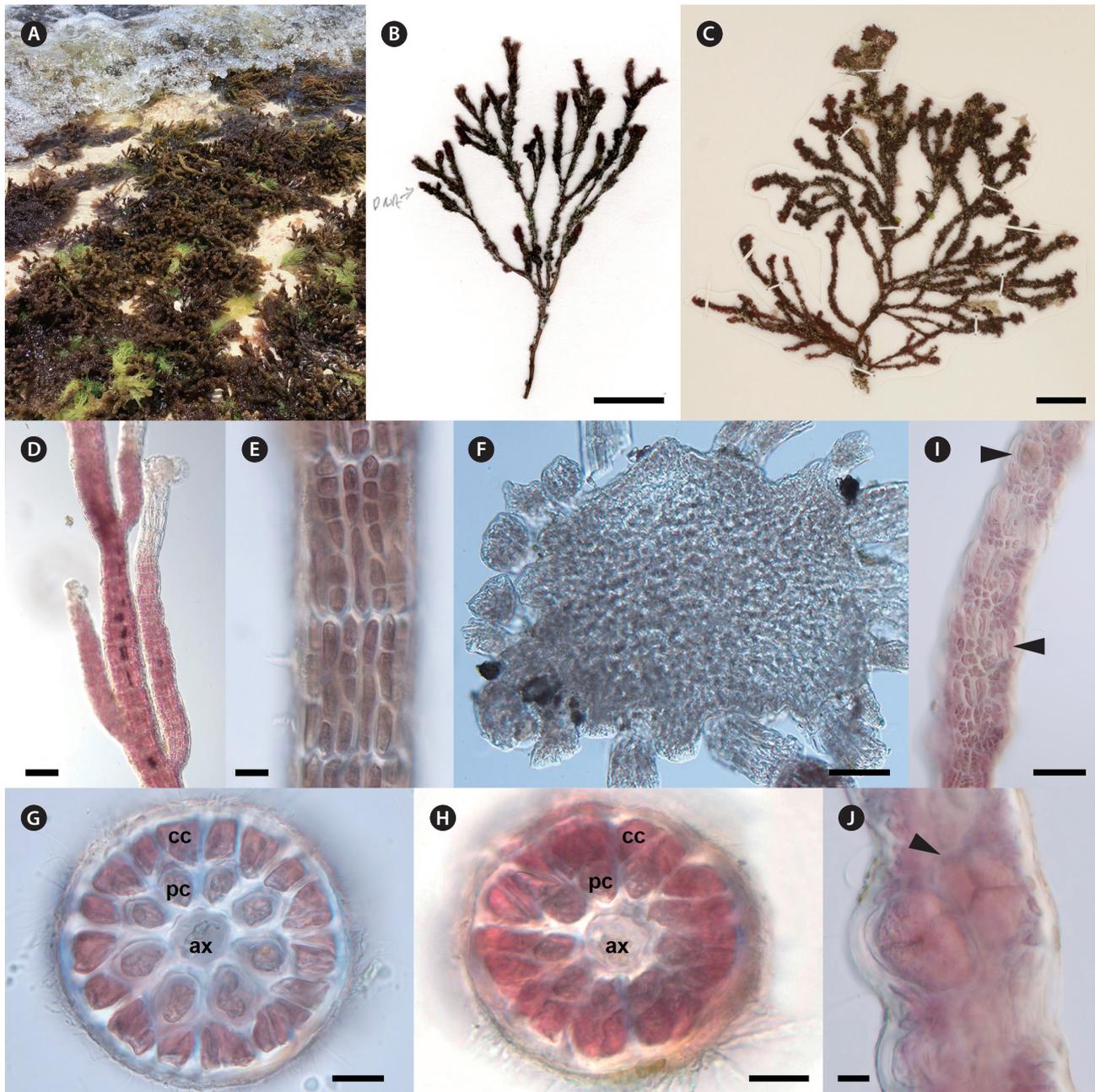
**Description.** Plants bushy, up to 5 cm high, tough and cartilaginous; axes widely di-, trichotomous or irregularly laterally branched (Fig. 2A & B); axes denuded at base or sparsely covered with wiry branchlets, 1.9 mm wide; branches usually densely (or occasionally sparsely) clothed with slender, stiff, usually simple determinate branchlets, 2-3 mm long and 98-270  $\mu\text{m}$  wide (Fig. 2D); primary axes sometimes denuded in lower portions; attached by a discoid holdfast which often spreads and may possibly coalesce with other holdfasts; branchlets polysiphonous, with axial cells 30-60  $\mu\text{m}$  in diameter, 10 or rarely 8 pericentral cells surrounded by up to 30 cortical cells in one layer (Fig. 2E); surface cortical cells irregular, in packets of 2-4 cells (Fig. 2C); apices with short vegetative trichoblasts remaining during development (Fig. 2F); reproductive structures not found.

**Holotype.** CNU037775 deposited in CNUK; a vegetative specimen; collected at Bahia de Kino (28°88'71" N, 112°04'96" W), Las Cruces, Sonora, Mexico on May 16, 2010 by Sung Min Boo.

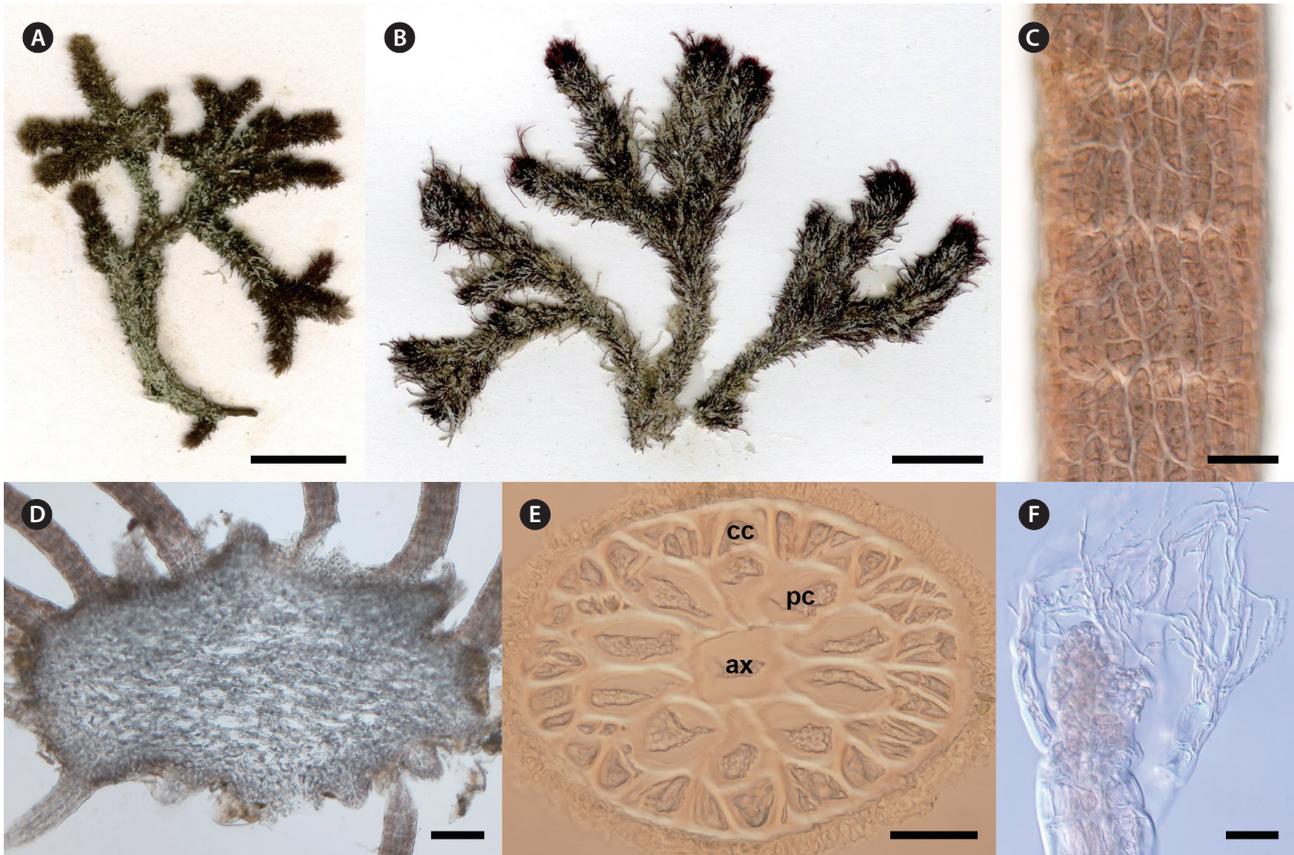
**Isotypes.** CNU037775-1, CNU037775-2, and CNU03775-3 in CNUK, Korea.

**Additional herbarium specimens observed.** CNU 058249 (Conquista Agraria, Baja California Sur, Mexico; Feb 27, 2014), CNU058249-2 (Conquista Agraria; Feb 27, 2014), CNU058291 (Cabo San Lucas, Baja California Sur, Mexico; Mar 1, 2014).

**Etymology.** The specific epithet honors the departed saint Rafael Riosmena-Rodríguez, previous Professor of



**Fig. 1.** *Digenea mexicana* sp. nov. G. H. Boo & D. Robledo. (A) Plants growing intertidally on sand covered rocks at Playa Xcalacoco, Quintana Roo, Mexico (type locality). (B) Type specimen (CNU080663). (C) Paratype specimen from Veracruz (MEXU 2326). (D) Alternate determinate branches (CNU080683). (E) Surface view of determinate branch showing cortical cell packets covering pericentral cells (CNU080681). (F) Transverse section of indeterminate branch showing radiating determinate branchlets (CNU080682). (G) Transverse section of determinate branchlet having eight pericentral cells (pc) around axial cell (ax) and a single layer of outer cortical cells (cc) (CNU080682). (H) Transverse section of determinate branchlet having nine pericentral cells (pc) around axial cell (ax) and a single layer of outer cortical cells (cc) (CNU080681). (I) Swollen determinate branchlet with tetrasporangia (arrowheads) (CNU080684). (J) Tetrahedrally divided tetrasporangia (arrowhead) (CNU080684). Scale bars represent: B & C, 2 cm; D, F & I, 100  $\mu$ m; E, G, H & J, 20  $\mu$ m.



**Fig. 2.** *Digenea rafaellii* sp. nov. G. H. Boo, G. Andrade-Sorcía & S. M. Boo. (A) Type specimen (CNU037775). (B) Paratype specimen from Conquista Agraria (CNU58249). (C) Surface view of determinate branchlet showing cortical cell packets covering pericentral cells (CNU037775). (D) Transverse section of indeterminate branch showing radiating determinate branchlets (CNU037776). (E) Transverse section of determinate branchlet having ten pericentral cells (pc) around axial cell (ax) and a single layer of outer cortical cells (cc) (CNU037775). (F) Apices with short vegetative trichoblasts (CNU037775). Scale bars represent: A & B, 1 cm; C, E & F, 50 µm; D, 200 µm.

Departamento de Biología Marina, Universidad Autónoma de Baja California Sur, La Paz, Mexico, for his outstanding contributions to the marine algae in Mexico.

**Distribution and habitat.** Presently confirmed in Bahía de Kino, Sonora and Cabo San Lucas, Conquista Agraria, and Las Conchas, Baja California Sur. Plants were collected on sand covered rocks in mid to low intertidal or sometimes in sublittoral zones.

**DNA sequences of type specimens.** For holotype, MH514879 (COI-5P) and MH514867 (*rbcL*); for isotype, MH514880 (COI-5P) and MH514868 (*rbcL*).

A morphological comparison of *D. mexicana* and *D. rafaellii* with all other species of *Digenea* is provided in Table 1.

### Mitochondrial COI-5P and plastid *rbcL* phylogenies

Twenty-four sequences were generated in this study: five of COI-5P and *rbcL* from each new species (here assigned to *D. mexicana* sp. nov. and *D. rafaellii* sp. nov.), respectively, and two each of COI-5P and *rbcL* for *Digenea* plants from Japan (Supplementary Table S1). All five plants of *D. mexicana* were identical in COI-5P and *rbcL*, and plants of *D. rafaellii* from four locations in the Gulf of California were also the same in both markers. *D. mexicana* differed by 3.6% in COI-5P and 2.5–2.7% in *rbcL* from *D. rafaellii* and by 3.0–3.4% in COI-5P and 0.98–1.07% in *rbcL* from *D. arenahauriens*. *Digenea* plants from Japan and Western Australia differed by 3.9–4.2% in

COI-5P from *D. simplex* from Tunisia.

The topology of the ML and BI trees was largely congruent, and only the ML tree is shown for COI-5P and *rbcL* (Figs 3 & 4). In the COI-5P phylogeny (Fig. 3), *D. mexicana* and *D. rafaellii* were distinct enough to be segregated from other species of *Digenea*. However, *Digenea* plants from Japan and Australia did not form a clade with

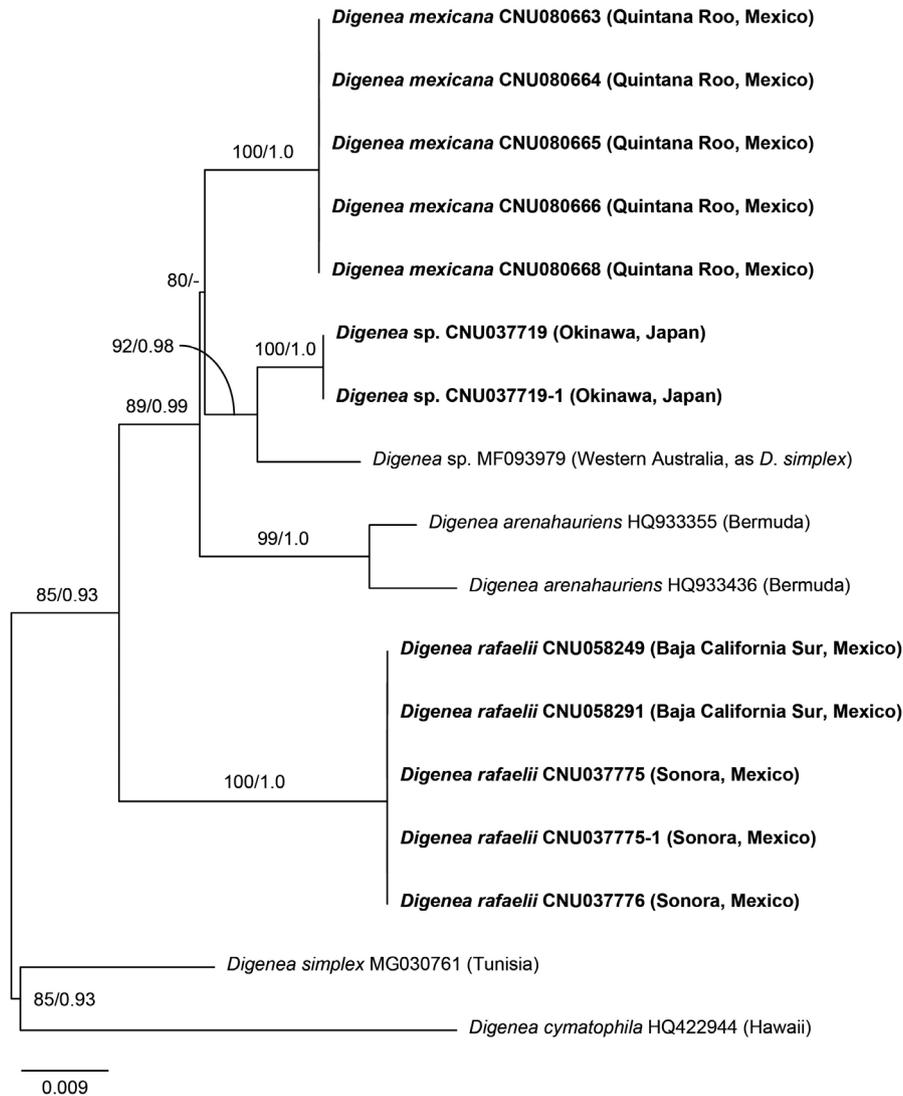
*D. simplex* from the Mediterranean.

In the *rbcL* phylogeny (Fig. 4), the monophyly of the genus *Digenea* was strongly supported (100% ML, 1.0 BPP). *D. rafaellii* from the Gulf of California was placed outside the group of *D. arenahauriens*, *D. mexicana*, and *D. simplex*. Phylogenetic relationships of *D. mexicana* with other species were not resolved.

**Table 1.** A comparison of *Digenea mexicana* and *D. rafaellii* with other species of *Digenea*

Feature	<i>D. mexicana</i>	<i>D. rafaellii</i>	<i>D. arenahauriens</i>	<i>D. cymatophila</i>	<i>D. simplex</i>	<i>D. subarticulata</i>
Type locality	Playa Xcalacoco, Quintana Roo, Mexico	Bahia de Kino, La Cruzes, Sonora, Mexico	Captain William's Bay, south shore of Bermuda Is., Bermuda	West end of Koko Head Parking, O'ahu Island, Hawaii	Trieste, Italy	Black rock, Kosi Bay, KwaZulu-Natal, South Africa
Habit	Mostly erect	Mostly erect	Mostly erect	Decumbent, small, axes arising on a layered prostrate crusts	Mostly erect	Mostly erect
Plant length	Up to 10.5 cm	Up to 5 cm	Up to 7.3 cm	Up to 3 cm	10-20 cm (-25)	Up to 2 cm
Axes	Cylindrical, up to 1.5 mm wide	Cylindrical, up to 1.9 mm wide	Cylindrical, up to 4.1 mm wide	Cylindrical, ca. 0.5 mm wide	Terete	Cylindrical, ca. 3 mm wide
Indeterminate branches	Irregular or widely dichotomous, rarely trichotomous	Irregular or widely dichotomous, rarely trichotomous	Dichotomous or irregular	Unbranched or branched once to a few times	Widely dichotomous	Little branched
Determinate branchlets	Simple to branched, up to 6.5 mm long	Simple, 2-3 mm long	Simple, 44-60 segments, up to 7.7 mm long	Simple, less developed	Simple, (19-) 29-49 segments	Simple, relatively at right angles to the bearing branch, articulated in upper portion, ca. 3 mm long
Surface cortical cell packets	2-4 cells wide	2-4 cells wide, irregularly	Four cells wide, 3-5 cells in central	Arranged in groups	Two, rarely three, cells wide, parallel rows	1-3 cells wide, irregularly
Outer cortical cells	One layered (ca. 20 cells in tier)	One to two layered (ca. 30 cells in tier)	One layered (17-34 cells in tier)	One to two layered	One layered (14-20 (-24) cells in tier)	Two layered (ca. 20 cells in tier)
Pericentral cells	8, rarely 9	10, rarely 8	(8-) 9-12 cells	10-12 cells	7-10 cells	10-12 cells
Trichoblasts	Deciduous, developed on apices	Deciduous, developed on apices	Deciduous, developed on apices	Common, colorless, pinnately branched	Deciduous, developed on apices	NA
Tetrasporangia	On inflated determinate branchlets, tetrahedral, 40-70 µm diam.	NA	On distorted determinate branchlets, tetrahedral, 38-82 µm diam.	Spirally arranged along branchlets, tetrahedral, ca. 100 µm diam.	One per swollen cell, on distorted determinate branchlets, tetrahedral, 75-100 µm diam.	Immersed in distorted determinate branchlets, tetrahedral
Cystocarps	NA	NA	Two or more per branchlet, urceolate, 390-500 µm diam.	One per branch, urceolate, 300 × 275 µm	Ovoid	NA
Spermatangia	NA	NA	NA	Forming capitula on branch tips	Forming flat, oval discs	NA
References	This study	This study	Schneider et al. (2018)	Norris (1994) (as <i>Alsidium cymatophilum</i> )	Kylin (1956), Cabioc'h et al. (2006), Schneider et al. (2018)	Simons (1970)

NA, not applicable.



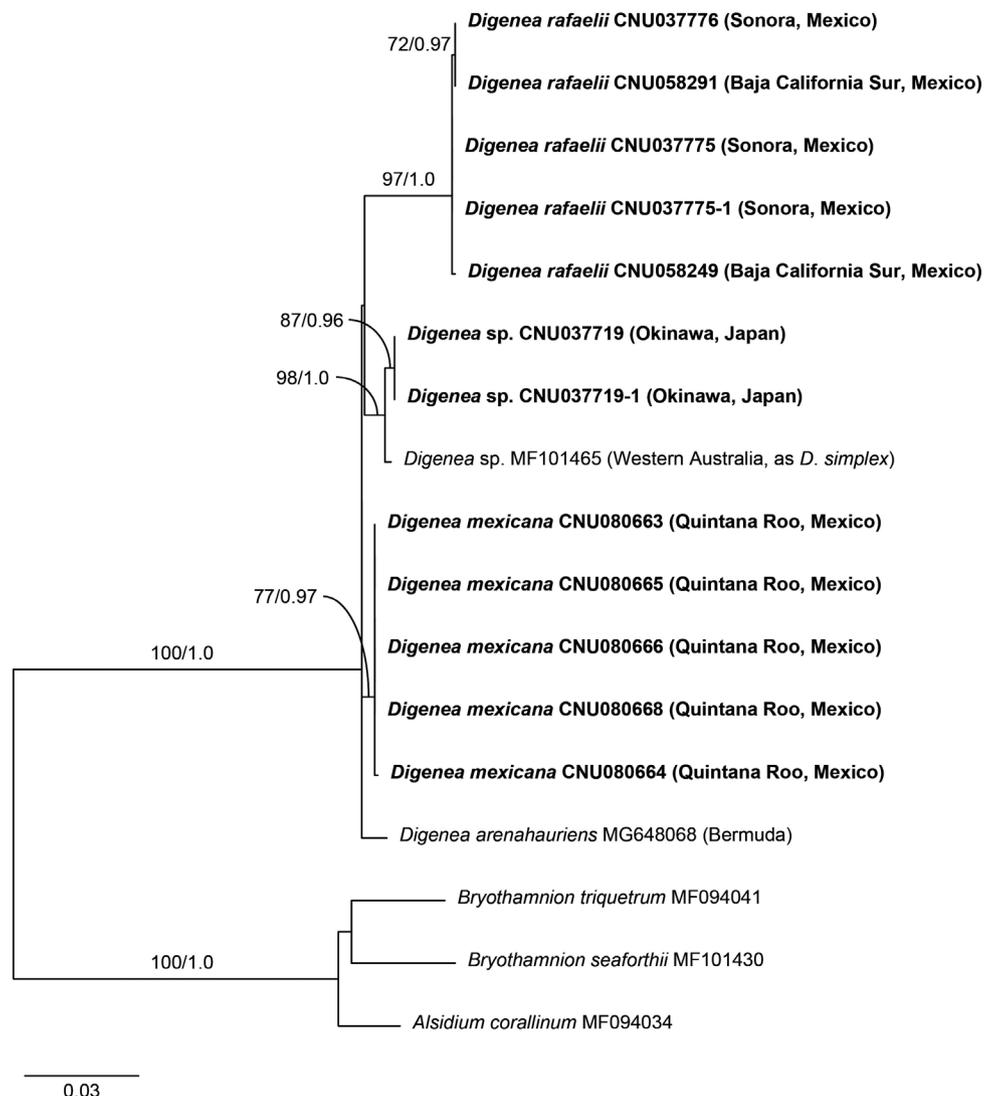
**Fig. 3.** Maximum likelihood (ML) tree inferred from mitochondrial COI-5P sequences of the genus *Digenea*. ML bootstrap values ( $\geq 50\%$ ) and Bayesian posterior probabilities ( $\geq 0.90$ ) are shown at branches. Dashes indicate values  $< 50$  or  $< 0.90$ . Bold letters indicate newly generated sequences in this study.

## DISCUSSION

Our results indicate that, based on COI-5P and *rbcL* sequences, the *D. simplex* complex comprises more than six genetically distinct species, including *D. mexicana* sp. nov. and *D. rafaелиi* sp. nov. from Mexico. Interestingly, *D. mexicana* on the Caribbean side is geographically isolated from *D. rafaелиi* on the Pacific side, as the other species are endemic to their distributional range. All species can be recognized by comparative morphology (Table 1). The habit and size of plants, the presence or absence of indeterminate branches, the number of pericentral cells and the surrounding outer cortical cells, and branching of de-

terminate branchlets can be used as diagnostic characters in segregating species, as discussed below. However, identification of *Digenea* species without DNA data and / or information on collection sites could prove difficult.

*D. mexicana* is distinct from *D. rafaелиi* in morphology as well as COI-5P and *rbcL* sequences. It occurs at Playa Xcalacoco, Quintana Roo on the Yucatan Peninsula. Plants grow to 10.5 cm tall and have cylindrical axes, irregular or widely dichotomous branches of indeterminate growth, simple to branched branchlets of determinate growth, eight pericentral cells in determinate branchlets and tetrasporangia formed in the upper inflated branchlets. Plants from Veracruz are large (about 14.5 cm tall in



**Fig. 4.** Maximum likelihood (ML) tree inferred from plastid *rbcL* sequences of the genus *Digenea*. ML bootstrap values ( $\geq 50\%$ ) and Bayesian posterior probabilities ( $\geq 0.90$ ) are shown at branches. Dashes indicate values  $< 50$  or  $< 0.90$ . Bold letters indicate newly generated sequences in this study.

herbarium specimen of MEXU 2326), as comparable in size to the Mexican plant illustrated by Dreckmann and Sentías (1994). Tetrasporangia were formed on the distal part of determinate branchlets, as reported in previous studies (Dawson 1963, Dreckmann and Sentías 1994, Norris 2014). Mexican plants have a monophasic life history including only a tetrasporangial phase (Dreckmann and Sentías 1994).

*D. rafaелиi* occurs at four locations on the south and north coasts of the Baja California Peninsula. Plants grow to 5 cm tall and have cylindrical axes, irregular or widely dichotomous branches, and simple determinate branchlets with ten pericentral cells. The size of *D. rafaелиi* is

much smaller than that of *D. mexicana*. Plants from Baja California Peninsula have been reported to grow to 25 cm tall (Norris 2014), comparable to that of European plants of *D. simplex* (Kylin 1956, Cabioc'h et al. 2006), however plants in our collections are usually about 5 cm tall. Our specimens from Puerto Peñasco (collected in May 2010) and La Paz (collected in February 2015) were sterile without tetrasporangia and sexual reproductive structures. Populations of *Digenea* from the Pacific coasts of Mexico are mostly vegetative in the present and previous collections (Dreckmann and Sentías 1994), although tetrasporangia were present only in specimens at Bahia de Kino in August and September (Mendoza-González and

Mateo-Cid 1986). We could speculate that Baja California coasts is the northern boundary of *D. rafaellii*, where its populations might be maintained mostly by vegetative reproduction or mitotic tetrasporangia. Range of *D. rafaellii* might be extended to Islas Revillagigedo and Costa Rican Golfo Dulce, where *Digenea* plants commonly occur (Taylor 1945, Dawson 1957).

*D. arenahauriens* (type locality: Captain William's Bay, Bermuda) grows to 7.3 cm tall and has terete main axes with long wiry determinate branchlets composed of 44-60 axial segments (Schneider et al. 2018). Determinate branchlets grow to 7.7 mm long and have a relatively large axial cell (27-78 µm in diameter) with 9-12 pericentral cells surrounded by 17-34 outer cortical cells. Female plants have two or more cystocarps borne on a determinate branchlet. Tetrasporangia are formed subapically on determinate branchlets, are tetrahedrally divided and 38-82 µm in diameter (Schneider et al. 2018). *D. arenahauriens* occurs in Bermuda and the Caribbean Antilles (Schneider et al. 2018). Further study of *Digenea* in the Caribbean Sea will highlight the speciation and biogeographic patterns of *D. arenahauriens* and *D. mexicana*.

*D. cymatophila* (type locality: O'ahu Island, Hawaiian Islands) was transferred from *Alsidium* based on plastid genome data (Díaz-Tapia et al. 2017). It occurs on rocks in sandy beaches, forming a mass of erect determinate branches from a layered basal crust (Norris 1994). It develops only one cystocarp on each fertile branchlet of female plants. *D. cymatophila* is the sole decumbent species in *Digenea*, and the decumbent habit likely arose as a response to grazing pressure by herbivores (Lewis et al. 1987).

*D. simplex* (type locality: Trieste, Italy; the inner coast of Adriatic Sea) grows to 25 cm tall and has terete axes of indeterminate growth that are irregularly to dichotomously branched (Kylin 1956, Schneider et al. 2018). Determinate branchlets are wiry, with 29-49 segments and a relatively small axial cell (19-38 µm in diameter) and 7-10 pericentral cells surrounded by 14-20 (-24) outer cortical cells (Kylin 1956, Schneider et al. 2018). It occurs throughout the year in the Mediterranean except the Gulf of Lion (Cabioc'h et al. 2006).

*D. subarticulata* (type locality: Black Rock, Kosi Bay, KwaZulu-Natal, South Africa) is small in size (less than 2 cm tall) and has simple axes with 10-12 pericentral cells (Simons 1970). Determinate branchlets are relatively long and slightly articulated at the upper portion (Simons 1970). De Clerck et al. (2005) noted that *D. subarticulata* is likely representative of *Bryothamnion*, because of its

small size and occurrence apart from northern KwaZulu-Natal, where is the southern boundary of *Digenea*.

Both COI-5P and *rbcL* phylogenies indicate that *Digenea* plants from Japan and Australia, in the past referred to *D. simplex* (Yoshida 1998, Huisman and Borowitzka 2003), are different species from *D. simplex* from Tunisia. Considering that the type locality of *D. simplex* is Trieste, Mediterranean samples could be considered as typical. The close relationships (1.5% in COI-5P, 0.4% in *rbcL*) between the Japanese and Australian taxa suggest their recent divergence from a common ancestor, which has likely occurred widely from southern to northern hemispheres. Additional study is needed to unravel the phylogenetic bridge between Western Australia and Japan and to decide whether plants from Australia and Japan belong to the same species or are distinct species.

We dispute the merging of New Caledonian *Digenea vieillardii* Kützing into *D. simplex* by De Toni (1903) and Millar and Prud'homme van Reine (2005), who considered the former species as a heterotypic synonym of the latter. Kützing (1865) described *D. vieillardii* on the basis of specimens that Eugène Vieillard, a French botanist, collected during his expedition to New Caledonia. The diagnosis was "*Ramellis ramosis*", and the branched determinate branchlets were well illustrated in Tabulae 28 g, h, I, k, l in the protologue (Kützing 1865). Our present study supports the taxonomic value of branched determinate branchlets in *Digenea* because it characterizes *D. mexicana* from *D. rafaellii*. *D. vieillardii* has tetrachotomous to verticillate branchlets at the upper part, while determinate branchlets of *D. mexicana* are mostly formed once. In contrast, *D. simplex* has simple, unbranched determinate branchlets (Kylin 1956, Schneider et al. 2018). Molecular analysis of type or topotype material of *D. vieillardii* is necessary to unravel its phylogenetic relationship within *Digenea*.

This is the first study of the genetic discontinuity between *Digenea* plants from Mexico, where we describe two new species, *D. mexicana* in Yucatan Peninsula and *D. rafaellii* in the Gulf of California. The previous reports of *D. simplex* in Australia and Japan (Yoshida 1998, Huisman and Borowitzka 2003) are misidentifications due to the cryptic nature of the *D. simplex* complex. Further global sampling will highlight taxonomy and distribution patterns of the *D. simplex* complex. Since *D. simplex* is one of the major medicinal red algae (Ebadi 2006), our finding will contribute to its medicinal exploitation and the conservation of local species.

## SUPPLEMENTARY MATERIAL

**Supplementary Table S1.** Information on specimens included in molecular analyses (<http://e-algae.org>).

## ACKNOWLEDGEMENTS

We thank Rafael Riosmena-Rodriguez for his help in collection in Baja California Peninsula, José Luis Godinez-Ortega from MEXU for herbarium on loan, and Marina Morabito for COI-5P sequence of the Tunisian sample. The first author thanks field guiders in the pre-congress tours of 2009 IPC in Okinawa, Japan. This study was supported by a postdoctoral scholarship from the Silva Center for Phycological Documentation, University Herbarium, University of California Berkeley to GHB, PN-Conacyt: 2015-01-575 to DR, and a grant of Marine Biotechnology Program (2017143), Korean Ministry of Oceans and Fisheries to SMB. We appreciate John Huisman and anonymous reviewers for improving our manuscript.

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