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Reinstatement of *Phrix* (Delesseriaceae, Rhodophyta) based on DNA sequence analyses and morpho-anatomical evidence

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Abstract

Living material of a marine red alga consisting of a prostrate basal system and small erect blades was isolated from laboratory-incubated substratum collected in a sea cave at 7 m depth on Easter Island in the Southeast Pacific. The alga in culture was morphologically identified as *Apoglossum gregarium* (E.Y. Dawson) M.J. Wynne. Molecular analyses (*rbc*L, LSU, COI) revealed that this alga was not closely related to *Apoglossum ruscifolium* (Turner) J. Agardh, the generitype. It also was genetically and morphologically distinct from *Paraglossum* and other genera in the Delesseriaceae. Therefore, it is proposed that the genus *Phrix* J.G. Stewart be reinstated. Because the taxonomic synonym *Membranoptera spatulata* E.Y. Dawson has priority over *Hypoglossum gregarium* E.Y. Dawson, the binomial *Phrix spatulata* (E.Y. Dawson) comb. nov. is effected as the name for the sole species currently recognized in this genus of the Delesseriaceae (Rhodophyta). This is a new record of the species from Chile. The basal system in culture developed from numerous coalescent uniseriate filaments from which the blades arose and showed this isolate to be a male with spermatangial sori.

Key words: taxonomy, nomenclature, marine flora, Chile

Introduction

The genus *Phrix* of the red algal family Delesseriaceae (Order Ceramiales) was described by Stewart (1974), with the type and only species *Phrix gregarium* (E.Y. Dawson) J.G. Stewart, based on the species *Hypoglossum gregarium* E.Y. Dawson first described from the type locality of Isla San Lorenzo del Norte, Gulf of California, Mexico (Dawson 1966). Wynne (1985) presented reasons to transfer *P. gregarium* to the genus *Apoglossum* J. Agardh (1898) primarily because of the strong similarity in the apical organization and the fact that the lateral pericentral cells remain transversely undivided, as in *Apoglossum*.

Despite the small stature of this algal species (less than 10 mm in height) and its usual deep sublittoral habitat, *Apoglossum gregarium* has been reported to occur from scattered tropical, subtropical, and warm temperate locations, including southern California (Stewart 1974, 1989), Puerto Rico (Ballantine & Wynne 1985), Kwazulu-Natal, South Africa (Wynne & Norris 1991), Italy (Sartoni & Boddi 1993), the Mediterranean coast of Spain (Clavell & Polo 1998, 2000), Hawaii (Abbott 1999), Bermuda (Schneider 2000), the State of São Paulo, Brazil (Horta & Oliveira 2001), Korea (Park *et al.* 2006), Greece (Tsiamis & Bellou 2010), Venezuela (Gómez *et al.* 2013), and the northern Gulf of California, Mexico (Norris 2014). It is an easily recognized species because the usually simple blades often arise from a shared prostrate stolon and have a ruffled, undulate appearance, and they have a conspicuous uncorticated midline. In addition, tetrasporangiate plants bear tetrasporangial sori straddling the blade midline in an uninterrupted pattern, and the male plants bear fusiform-shaped spermatangial sori separated by lateral veins (Stewart 1974, Wynne 1985).

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Phylogenetic analyses have not previously been published on this species. We have examined the relationships of a culture isolate from Easter Island, where the species has not so far been recorded, with other Delesseriaceaen algae using plastid ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L), nuclear large subunit ribosomal DNA (LSU nrDNA), and mitochondrial Cytochrome c oxidase subunit I (COI). Our analyses showed it to be genetically distant from the type species of *Apoglossum* and other recognized genera of the Delesseriaceae.

Materials and Methods

Collection and culture

On 29 April 2014 FCK collected, using a sterile tube, a few cm^3 of coarse coral sand from the bottom of a sea cave, 7 m depth, at La Cathédrale, west coast of Easter Island, Southeast Pacific (109° 26'W; 27° 09'S). Easter Island lies in the Chilean Marine National Park Nazca-Desventuradas. The substratum sample was sent to AFP for incubation and isolation of emerging macroalgae following Peters *et al.* (2015). A minute red algal blade developing on a small pebble was isolated and served to generate a unialgal culture (code IP14-38), which was used for all further studies. The strain is deposited in the Culture Collection of Algae and Protozoa (CCAP) under the accession 2375/1, herbarium specimens made from culture material are deposited in SGO (168444), MICH (701906), MEL (2416394) and MNHN (PC0787947).

AFP air-shipped copies of the culture to LAM and JAW for further scrutiny. The package to JAW was shipped on 20 August 2016 and arrived on 3 September 2016. Although the blades in IP14-38 were 99% dead, a subculture (code JAW4876) was placed in 80 mL of low nutrient Modified Provasoli's ES medium (West 2005) (5 mL enrichment per 1 L sterile seawater at 30‰ salinity) with 2 drops of 1% germanium dioxide to control diatoms and about 1 mg of powdered Na Penicillin G to control cyanobacteria. The 50 × 70 mm Pyrex dishes were maintained in stationary condition at 10:14 light-dark daily photoperiod, 19–22°C, and 10–11 µmol photons m^{-2} s^{-1} cool white LED lighting for several weeks while recovery, growth and reproduction were followed. A higher irradiance (15–20 µmol photons m^{-2} s^{-1}) enhanced development. Further subcultures were placed on a rotary shaker at about 70 rpm with irradiance of 10–12 µmol photons m^{-2} s^{-1} to also enhance growth.

Molecular phylogenetic analyses

The DNA extraction from live material was as described by Hayakawa *et al.* (2012) or by using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturers' protocol, improved by a CTAB pre-treatment according to Gachon *et al.* (2009). The primer sequences to amplify and sequence almost the entire *rbc*L, *psa*A and *psb*C, partial LSU nrDNA and 5'partial COI are presented in Table 1. The PCR reactions were in a total volume of 25 µL cotaining about 40 ng of template DNA, 0.4 µM of each primer, 0.25 mM of each dNTP, 1x PCR buffer and 0.5 units of KOD FX neo DNA polymerase (Toyobo, Osaka, Japan). The thermal profile for PCR was as follows: initial denaturation at 94°C for 2 min, 30 cycles at 98°C for 30 s, 50°C for 30 s and 68°C for 90 s, and final extension at 68°C for 7 min. Excess primers and dNTP were removed from the PCR products using Exosap-it (Affymetrix, Santa Clara, CA). Because nonspecific PCR products were detected in the LSU amplification, the appropriate band was separated on 1.5% agarose gel and purified using Qiaex II agarose-gel extraction kit (Qiagen). DNA sequencing was performed by Eurofins Genomics (Tokyo, Japan) and Source Bioscience (Livingston, UK) and the sequences were deposited in DDBJ/ENA-Bank/GenBank with the accessions LC311653-5 (*rbc*L, COI, LSU, respectively) and LC363848-9 (psaA and psbC, respectively).

The acquired LSU rDNA sequences were aligned using the MAFFT ver. 7 online application (http://mafft.cbrc.jp/alignment/software/) based on the L-INS-i algorithm with default parameters, which reflected RNA secondary structure. Then adjustments of the resulting alignments were performed manually (final length was 1,393 bp). No indels were present in *rbc*L (final length was 1,333 bp) and COI sequences (final length was 664 bp). The models for LSU rDNA and each codon of *rbc*L and COI were selected using the program Kakusan4 (Tanabe 2011) (Table S1).

Published sequences from members of the Delesserioideae were included in the phylogenetic analyses and two species of the Nitophylloideae were used as outgroup. Maximum Likelihood (ML) trees were constructed using the TREEFINDER ver. March 2011 software (http://www.treefinder.de) and were evaluated by bootstrap analysis with 1,000 replications. For Bayesian Inference (BI) analysis, Markov chain Monte Carlo (MCMC) iterations were conducted for 5,000,000 generations, with sampling every 100 generations using MrBayes 3.2.6 (http://mrbayes.sourceforge.net). Two independent simultaneous MCMC runs were performed to assess appropriate chain mixing

and to ensure topological convergence (split frequency ≤0.01). After inspection of convergence and stabilization with Tracer 1.5 (http://tree.bio.ed.ac.uk/software/tracer), the first 10% of generations were discarded as burn-in and clade posterior probabilities were computed from the remaining trees. Phylogenetic analyses were performed on the three markers separately. We have not analyzed the combined data set because the numbers of both LSU and COI sequences of delesseriacean species are limited.

The sequences of *psa*A (1566 bp) and *psb*C (1010 bp) were not used in phylogenetic analyses because of unavailable sequences of close relatives. They were compared with other sequences using BLAST (Zhang *et al.* 2000).

TABLE 1. Oligonucleotide primers used for PCR and sequencing.

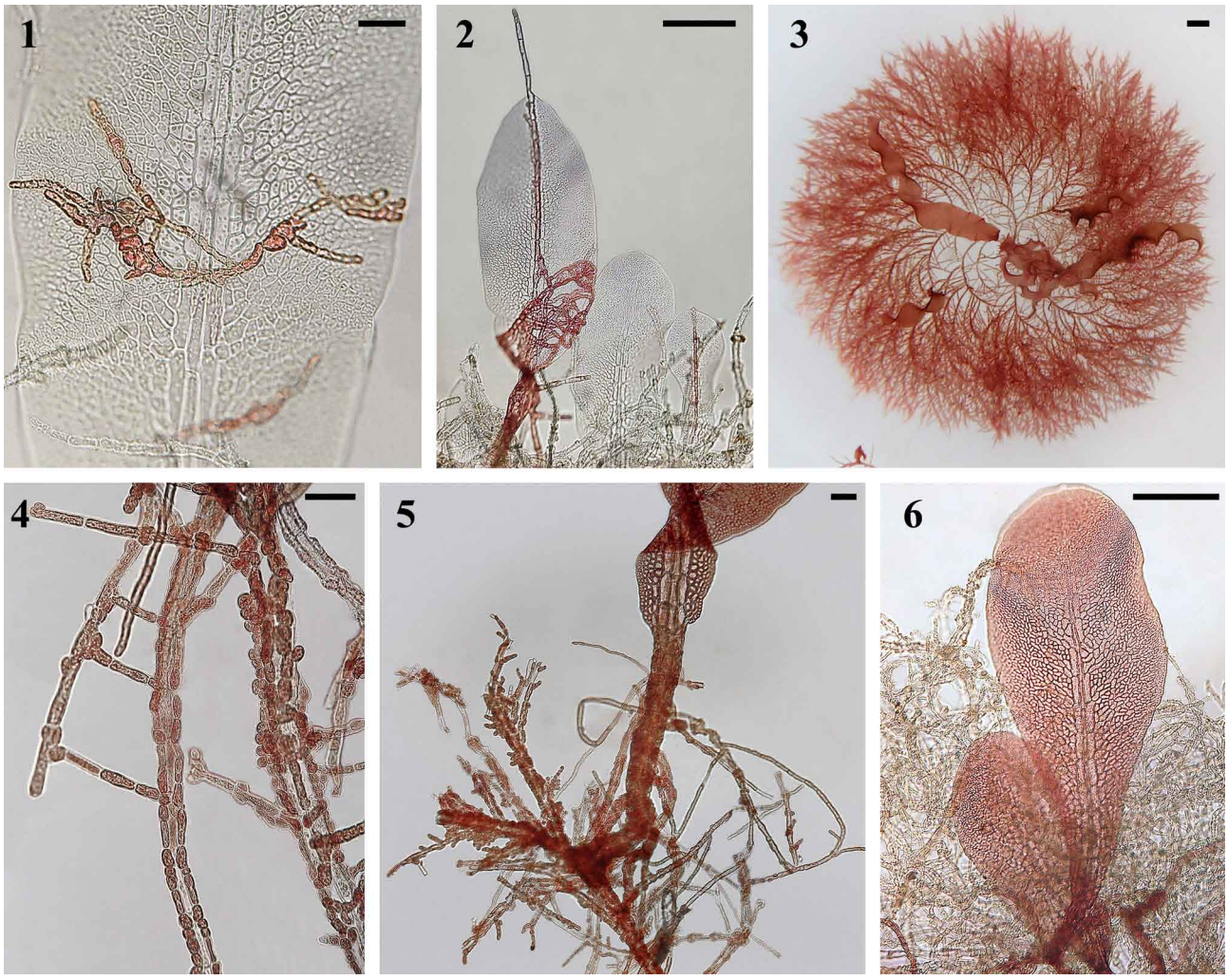
Gene to anneal	Primer name	Primer sequence (5′–3′)	Direction (F/R)	Reference
*rbc*L	rbc–F3	ATGTCTAACTCTGTAGAAGAACGGAC	F	Present paper
*rbc*S	rbc–R4	CCTCTWGTATTATCAAAWGCRTT	R	Present paper
*rbc*L	rbc–R6	CCAATWGTACCWCCWCCRAAYTG[1]	R	Present paper
*rbc*L	rbc–F7	GGTATYCAYTGYGGWCARATGC[1]	F	Present paper
*rbc*L	rbc–R8	ATCTYTCTTTCCARCGCATRAAWGG[1]	R	Present paper
*rbc*L	rbcL-P2	GAWCGRACTCGAWTWAAAAGTG	F	Kawai *et al.* (2007)
*rbc*L	Ral-R952	CATACGCATCCATTTACA	R	Kawai *et al.* (2007)
*rbc*L	rbcL1273F	GTGCGACAGCTAACCGTG	F	Peters *et al.* (2004)
*rbc*S	rbcS139R	AGACCCCATAATTCCCAATA	R	Peters & Ramírez (2001)
*psa*A	psaA130F	AAACWACWACTTGGATTTGGAA	F	Yoon *et al.* (2002)
*psa*A	psaA940R	TATGDCCAATWCCCCAATT	R	Bittner *et al.* (2008)
*psa*A	psaA870F	GGNGGWYTATGGTTAAGTGA	F	Yoon *et al.* (2002)
*psa*A	psaA1760R	CCTCTWCCWGGWCCATCRCAWGG	R	Yoon *et al.* (2002)
*psb*C	psbCF	GTGGAAACGCCCTTTAATA	F	Draisma *et al.* (2010)
*psb*C	psbCmidR	CCYCCWACDARATCTTCCATATTATC	R	Draisma *et al.* (2010)
*psb*C	psbCR1	TTAATCAATTGGACGCATTG	R	Draisma *et al.* (2010)
LSU	LSU–5F	GCATATAATTAAGCGSAGGAAAAGAAAC	F	Present paper
LSU	LSU–ZR	TGATAGGAAGAGCCGACATCGA	R	Harper & Saunders (2001)
LSU	LSU–YF	GCAGGACGGTGGCCATGGAAGT[1]	F	Harper & Saunders (2001)
LSU	LSU–4R	GCCCTGAAAATGGATGRCGC[1]	R	Present paper
COI	COI-1F	TTTTCAACTAAYCAYAAAGATATAGG	F	Present paper
COI	COI-1R	AAACTTCAGGATGACCAAAAAAYCA	R	Present paper

[1] These primers were only used for sequencing.

Results

Culture and morphology

In subculture 4876 very few living cells were seen initially in the bleached blades. After 12 days these cells began elongating, dividing and branching between dead blade cells. Live cell shapes usually conformed to the space between dead cells (Fig. 1). Once the filaments emerged freely in the culture medium the cells became more uniform and longer (Fig. 2). A basal system of coalescent rhizoidal filaments grew attached to the glass dish with blades arising from them (Fig. 3). This prostrate basal system developed from horizontal branching filaments attached to glass showing a levorotary growth pattern (Fig. 3). The attached system continued expanding outward and upward in the basal disc forming thick structures of numerous coalescent parallel filaments arising at the base of developing blades (Figs 4–6). Primary uniseriate filaments of the basal system had apical and intercalary cells 12–15 µm in diameter and 55–80 µm long. The uppermost cells of free filaments were uninucleate and pale with small plastids. Branching began from about the 6th intercalary cell from the apex, forming short lateral cells either singly or in opposite pairs that adhered closely to the axial filaments, remaining adherent and dividing as they elongated (right and middle shoots, Fig. 4). Some oblique laterals radiated outward from these adherent filaments and their apical cells adhered to adjacent uniseriate filaments that also developed adherent parallel filaments (left shoot, Fig. 4). At this stage the plastids developed full colour and filled cell perimeters. Apical and intercalary cells of adherent filaments also divided and fused with the axial filament cells forming secondary pit connections. The thickened (up to 200 µm diameter) structures arising from these coalesced filaments were enclosed by the cylindrical bases of the erect flattened and distinctly undulate monostromatic blades. The blades were usually unbranched (Figs 3, 6, 7), but secondary blades developed sometimes on the upper main blade margins (Fig. 9).



FIGURES 1–6. *Phrix spatulata* (E.Y. Dawson) comb. nov. **1.** Living branches ramifying through dead blade. Scale bar = 50 µm. **2.** Live filament extends through axial filament of dead blade and into culture medium. Scale bar = 150 µm. **3.** This filamentous basal system attached to glass was derived from a single excised filament. The levorotary growth pattern expands outward. At the center of the basal disc numerous coalescent parallel filaments occur at the bases of developing blades. Scale bar = 1 mm. **4.** Formation, coalescence and elongation of horizontal filaments. Shoot on right with central axial filament enclosed by parallel adherent branches bearing oblique uniseriate laterals projecting toward middle shoot also enclosed by parallel filaments. Middle shoot has 3 uniseriate laterals growing toward and attaching to uniseriate shoot on left. Scale bar = 100 µm. **5.** Basal system of radiating filaments developing into thick structure of adherent parallel filaments projecting up and around lower blade. Scale bar = 70 µm. **6.** Two young blades arising from thickened bases like that in Fig. 5 entangled with live filaments. Scale bar = 200 µm.

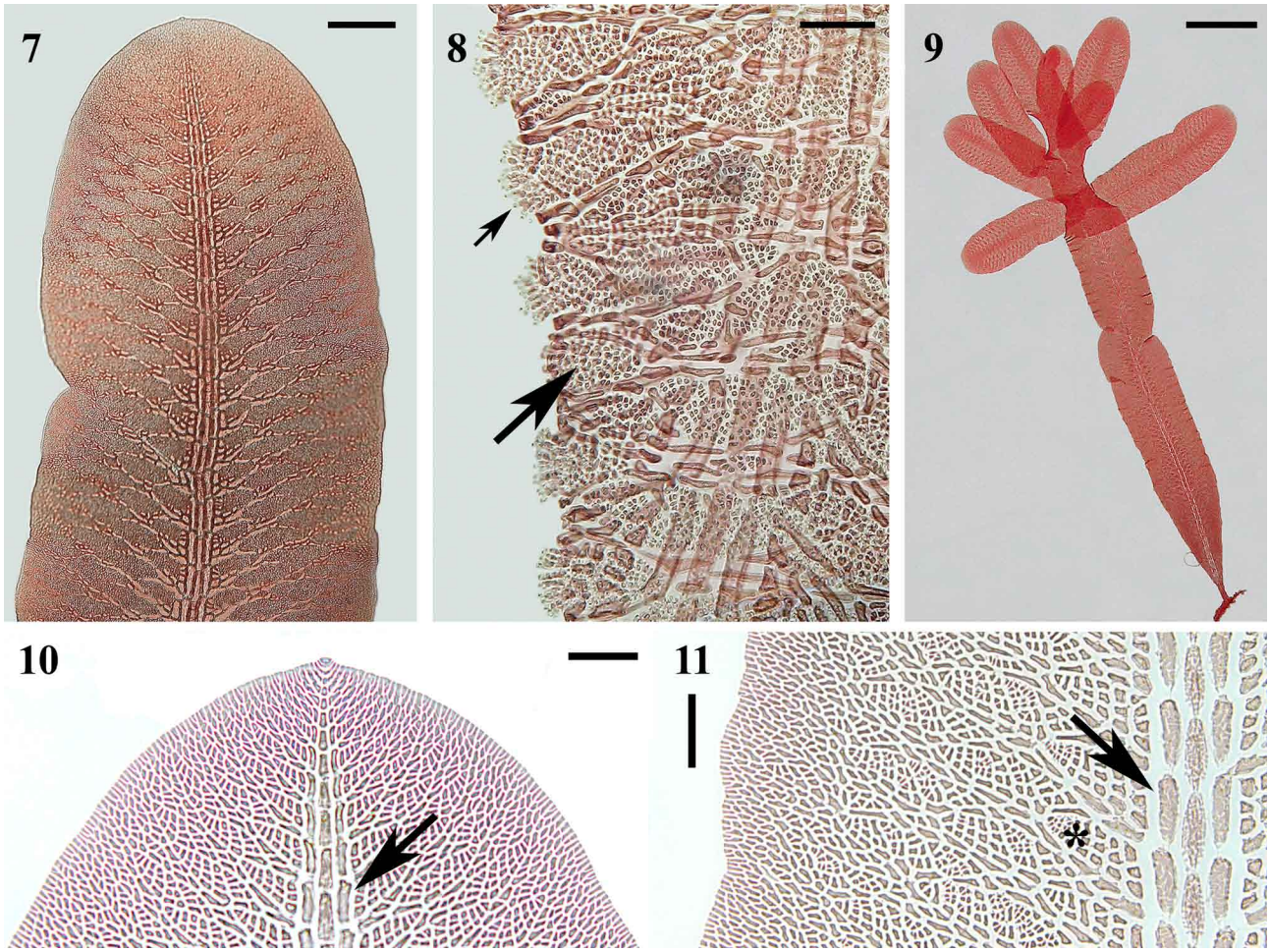
At 5.5 weeks in culture the blades about 860 µm wide had numerous fusiform spermatangial sori (140–190 × 40–50 µm) in parallel series of 3–6 between the lateral veins of the blade wings (Figs 7, 8). Blades developed more quickly in shaker culture. Images of this unknown alga, both from living (Figs 1–7) and fixed material (Figs 8, 10–11), were sent to MJW, who identified the alga as *Apoglossum gregarium* (E.Y. Dawson) M.J. Wynne.

Molecular results

The *rbc*L analysis (Fig. 12), using 31 sequences, revealed that the Easter Island isolate of *Apoglossum gregarium* (*Phrix spatulata* used in phylogenetic tree, see end of Discussion for nomenclature) made a clade with *Paraglossum* spp., however with only weak bootstrap support (61%). *Apoglossum ruscifolium* and *A. oppositifolium* (Harvey) J. Agardh clustered with *Loranthophycus californicus* E.Y. Dawson (Tribe Membranoptereae) (78% bootstrap value), resulting in the Tribe Apoglosseae as a paraphyletic group. The other taxa were even more distant. In the LSU tree (Fig. 13), for which 25 sequences were available, *Apoglossum ruscifolium* made a clade with *Paraglossum* spp. with 79% bootstrap support but the Easter Island isolate was in a weakly supported cluster (60% bootstrap value) with members of the Caloglosseae. In the COI tree (Fig. 14), calculated from 18 sequences, the isolate clustered with neither *Apoglossum*

ruscifolium nor the two available *Paraglossum* species and these apoglossaeans were resolved as a paraphyletic group. The other tribes made a cluster that was supported by a bootstrap value of 91%.

The sequences of *psa*A and *psb*C of *Phrix spatulata* were genetically distant (86–89% identity, respectively) from the available sequences of Delesseriaceae, which did not include members of the Apoglosseae. The strongest genetic similarity of the Easter Island isolate was with members of the Membranoptereae.



FIGURES 7–11. *Phrix spatulata* (E.Y. Dawson) comb. nov. **7.** Apex of blade with numerous fusiform spermatangial sori in series between lateral veins of the wings. Scale bar = 200 µm. **8.** Permanent mount slide showing folded blade margin and sori (one indicated by arrow) with branching of spermatangial mother cells and spermatangia (one indicated by small arrow). Scale bar = 50 µm. **9.** Excised mature blade 18 mm long with 6 secondary blades. Scale bar = 2 mm. **10–11.** Permanent mount slide showing apical (10) and middle (11) parts of blade. Note undivided pericentral cells (arrows show one in each Fig) as well as incipient spermatangial sori (one indicated by asterisk in Fig. 11). Scale bars = 50 µm.

Discussion

This is the first time *Apoglossum gregarium* was taken into unialgal culture and also the first time to generate sequences for the species. There is no previous record of this taxon from the southeast Pacific. Our isolate from Easter Island showed the morphological characteristics of *A. gregarium* (Stewart 1974, Wynne 1985), viz. presence of an extended basal system developing multiple erect blades (Fig. 3), small size and undulate margins of these spatulate blades (Figs 3, 8), an uncorticated midrib, undivided lateral pericentral cells, and spermatangial sori in patches between microscopic veins (Figs 8, 10, 11). Ecologically, the development of our isolate on substratum collected in an underwater cave also coincides with previous records of *A. gregarium* from the type locality and elsewhere from shaded habitats, usually in greater depth (for references see Introduction). We have therefore great confidence that our strain from Easter Island belongs to *A. gregarium* originally described from the Gulf of California.

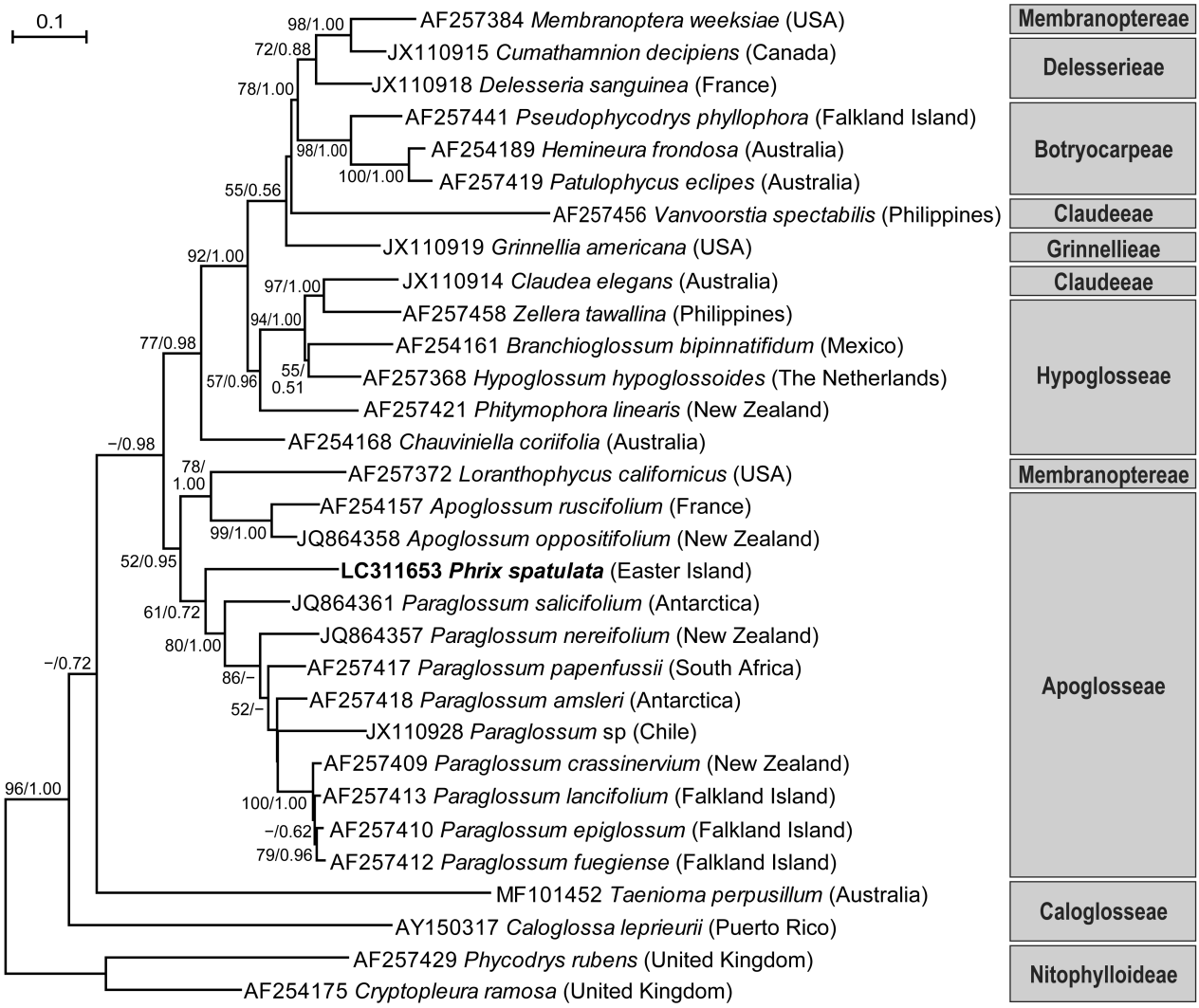


FIGURE 12. ML phylogeny of the Delesseriaceae inferred from partial *rbc*L gene sequences. Bootstrap values for ML (>50%; left) and posterior probabilities for BI (>0.5; right) are given on each branch. The scale is in units of nucleotide substitutions per site.

The basal system of this delesseriacean alga is similar to some others like *Polyneuropsis* in which filaments coalesce to form thick structures around bases of upright blades (Wynne *et al.* 1973) but very different from basal structures in others like *Hypoglossum rhizophorum* (Ballantine & Wynne 1988). Other authors have rarely described delesseriacean bases even though they are important in the alga's overall development and it is unknown how the transition from prostrate filaments to erect blades is regulated in this group of algae. *Apoglossum gregarium* may be an interesting species to unravel this transition more in detail, however for this it will be necessary to isolate tetrasporophytes and female gametophytes and to study the development starting from spores.

Our isolate from Easter Island was a male gametophyte producing only male reproductive structures. It did not show mixed-phase phenomena such as the occurrence of spermatangia and tetrasporangia on the same blade as reported by Park *et al.* (2006) in Korean material.

The tribe Apoglosseae was described by Lin *et al.* (2012). Wynne (2014) recognized the tribe to contain the genera *Apoglossum, Paraglossum*, and the parasitic genus *Apoglossocolax* (Maggs & Hommersand 1993). While the plastid *rbc*L analysis weakly suggested the present alga could be a basal member of the genus *Paraglossum* (Fig. 12), this was not supported by the nuclear LSU (Fig. 13) and mitochondrial COI data (Fig. 14). Blade anatomy also speaks against inclusion of the alga from Easter Island in *Paraglossum*. The fact that the lateral pericentral cells do not divide transversely (Figs 7, 8, 11, 12) is a basic, possibly conservative feature in the delesserioid red algae. The alga from Easter Island shares this character with *Apoglossum*, whereas *Paraglossum*, like *Delesseria*, possesses transversely divided pericentrals (Wynne 1984, Lin & Hommersand 2015).

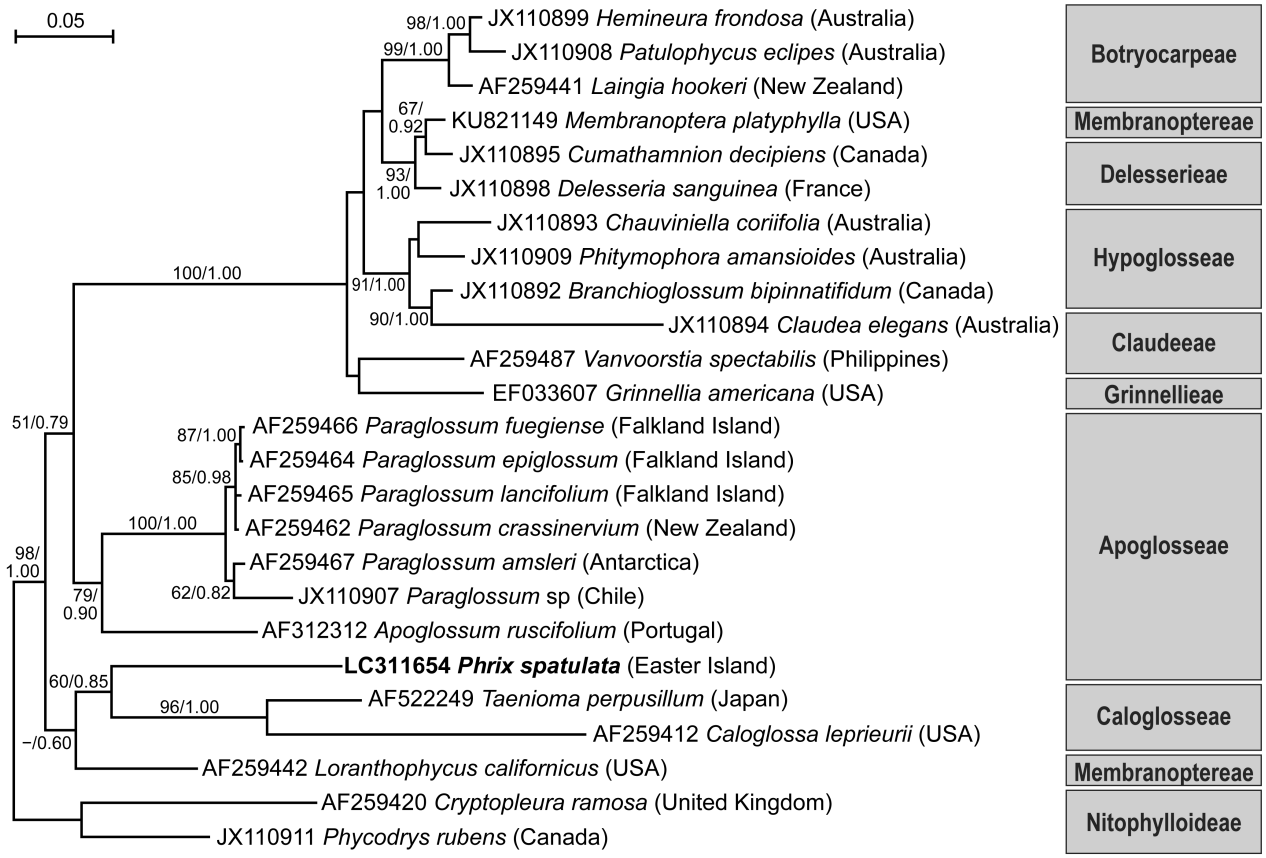


FIGURE 13. ML phylogeny of the Delesseriaceae inferred from partial LSU ribosomal DNA sequences. Bootstrap values for ML (>50%; left) and posterior probabilities for BI (>0.5; right) are given on each branch. The scale is in units of nucleotide substitutions per site.

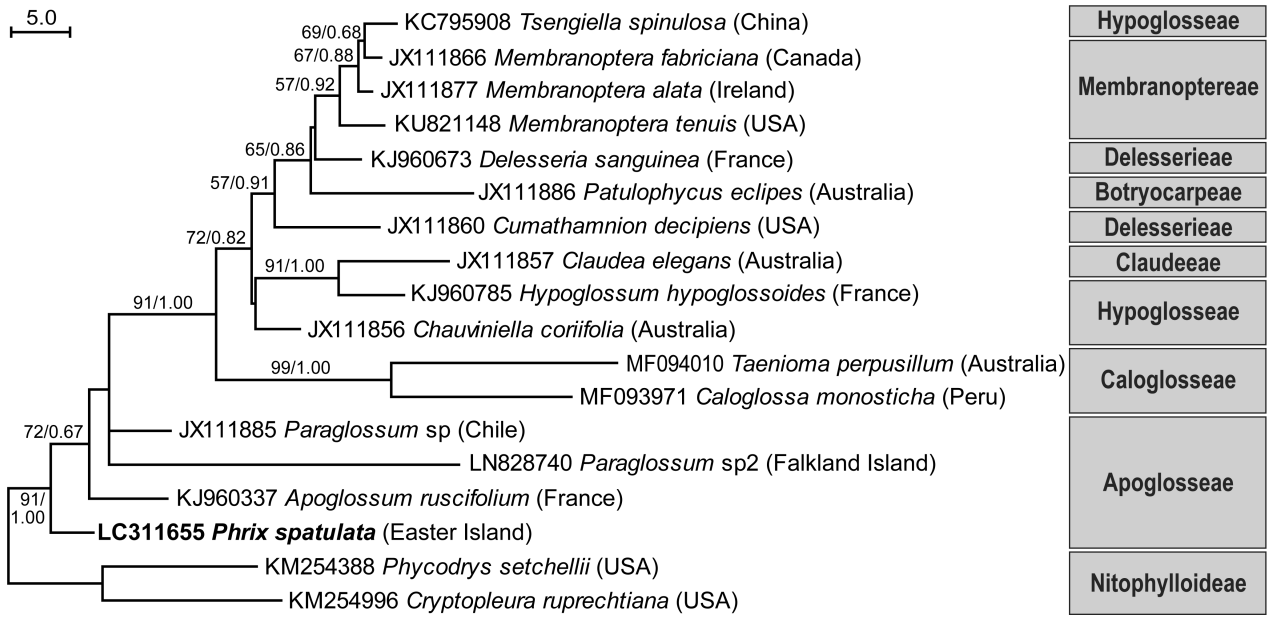


FIGURE 14. ML phylogeny of the Delesseriaceae inferred from partial COI gene sequences. Bootstrap values for ML (>50%; left) and posterior probabilities for BI (>0.5; right) are given on each branch. The scale is in units of nucleotide substitutions per site.

Biogeography also does not favor inclusion of the present alga in *Paraglossum,* which shows a circum-Antarctic distribution from polar (Antarctica) to temperate regions (Lin & Hommersand 2015). The only exception is *P. hancockii* (W.R. Taylor) M.J. Wynne from Galapagos Islands, however its phylogenetic position is not clear because of missing molecular data. An undescribed species of *Paraglossum* referred to as *Paraglossum* sp. GWS-2012b in the public DNA database (Wynne & Saunders 2012) from southern Chile, which we included in our analyses, was from cold-temperate

waters and was phylogenetically distant from our alga. *Apoglossum gregarium*, in contrast, has a subtropical to tropical distribution mostly in the northern hemisphere (see references in the Introduction). Using material not collected at the type locality for taxonomic conclusions is admittedly not optimal. However, because of the minute size of the alga and its deep sublittoral habitat our attempts to obtain living *A. gregarium* from the Gulf of California have so far been unsuccessful and historical preserved material was unavailable for molecular analyses. It is desirable that future studies corroborate our conclusions by using material of this taxon from (near) the type locality.

Total evidence indicates that our alga is phylogenetically distinct from both *Paraglossum* spp. and *Apoglossum* spp.. In consequence, we propose classification of *A. gregarium* in an own genus, leading to the resurrection of *Phrix,* proposed earlier for this species. Overall morphological similarity of our algae with *Apoglossum* spp. and the position in the *rbc*L tree (Fig. 12), which is based on the most comprehensive taxon sampling, suggest that *A. gregarium* is a member of the Tribe Apoglosseae.

When Wynne (1985) proposed treating *Phrix gregaria* as belonging to the genus *Apoglossum*, he presented evidence to regard *Membranoptera spatulata* E.Y. Dawson (Dawson 1950) as a taxonomic synonym. He pointed out that that name predated *Hypoglosssum gregarium* E.Y. Dawson (Dawson 1966). But because of the existence of the binomial *Apoglossum spathulatum* (Sonder) Womersley & Shepley (Womersley & Shepley 1982), Wynne chose the junior synonym *Hypoglossum gregarium* when he transferred the species of *Phrix* into *Apoglossum*. Reinstating the genus *Phrix* allows us to make use of the older species name *Membranoptera spatulata.*

Conclusions

The observations presented in the above account provides evidence for the reinstatement of the genus *Phrix* and for the transfer of the specific name with priority into this genus:

Phrix spatulata (E.Y. Dawson) M.J. Wynne, M. Kamiya & J.A. West comb. nov.
Basionym: *Membranoptera spatulata* E.Y. Dawson, *Amer. J. Bot.* 37(2): 157. 1950.
Heterotypic synonyms:
Hypoglossum gregarium E.Y. Dawson (1966: 65).
Phrix gregaria ["*gregarium*"] (E.Y. Dawson) J.G. Stewart (1974: 147).
Apoglossum gregarium (E.Y. Dawson) M.J. Wynne (1985: 169).

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