

The Minute Alga *Schizocladia ischiensis* (Schizocladiphyceae, Ochrophyta) Isolated by Germling Emergence from 24 m Depth off Rhodes (Greece)

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Received: 30 January 2020; Accepted: 10 March 2020; Published: 14 March 2020

Abstract: Substratum collected during diving surveys of sublittoral communities off the Greek island of Rhodes (Dodecanese, South-East Aegean) in late 2015 was incubated in the laboratory. Among the emerging macroalgal germlings, there was the second-ever record and isolate of the small benthic multicellular alga *Schizocladia ischiensis* of the poorly known monotypic Schizocladiphyceae, the sister group of the brown algae (Phaeophyceae). Its nuclear ribosomal small subunit, Rubisco spacer (*rbcL*, *psaA*, and *psbC* sequences (in total 5237 bp)) were similar to those of the only previous isolate of the species from Ischia, western Mediterranean. Our new strain formed branched upright thalli attached to the substratum by an amorphous substance secreted at the bottom of the basal cell. It is possible that *S. ischiensis* is a common member of the infralittoral and circalittoral communities in the Mediterranean and generally overlooked because of its minute size. Germling emergence appears to represent the method of choice to reveal benthic algae of this small size.

Keywords: macroalgae, molecular-assisted identification, phytobenthos

1. Introduction

Significant gaps remain in the knowledge of the seaweed flora of the Mediterranean, which is regarded as a species-rich region, with 190 green, 277 brown, and 657 red macroalgae recorded so far [1,2]. However, there are a large number of taxa in need of taxonomic reassessment, and recently revealed cases of cryptic diversity [3,4] suggest that molecular-assisted identification will be useful to improve our knowledge of this flora [2]. In general, there are more macroalgal records in the Western than in the Eastern Mediterranean [5]; however, it is unclear whether this is due to a lower

macroalgal diversity in this part of the Mediterranean Sea or reflects a general trend of fewer biodiversity studies in the Eastern in comparison to the Western basin.

In particular, the deep-water circalittoral macroalgal flora of the Mediterranean remains underexplored to this date. Due to the high transparency of the oligotrophic waters in this sea, macroalgae can exist in deep habitats, where coralligenous communities are the most common type of seaweed assemblages [6,7]. Another conspicuous community is formed by large brown macroalgae such as *Cystoseira* and *Sargassum* species thriving at depths between 20 and 50 m [8,9]. Populations of the Mediterranean endemic kelp *Laminaria rodriguezii* may occur even deeper (80–100 m or more, [10,11]), as well as the red alga *Sebdenia monnardiana* (60 m, [12]) or the invasive green alga *Caulerpa taxifolia* var. *distichophylla* (100 m, [13]). These new findings suggest that deep, circalittoral waters still remain an open field for biodiversity exploration.

The examples mentioned above concern large and conspicuous macroalgae, which are comparatively easy to detect by divers or from ROVs. Time constraints during sampling often exclude collection of smaller algae, although interesting and endemic species have been described from the Mediterranean, e.g., the phylogenetically interesting brown algae *Discosporangium mesarthrocarpum* (Meneghini) Hauck, *Choristocarpus tenellus* (Kützinger) Zanardini, and *Verosphacela silvae* Alongi, Cormaci et G. Furnari [14,15]. For such smaller algae from the sublittoral, we have recently employed the germling emergence technique, by which macroalgae are obtained from natural substratum incubated in the laboratory [16]. This method has been used to reveal the diversity of macroalgae and their microstages, e.g., [17–24]. We have now used the same method for substrata collected during diving surveys off Rhodes (Greece) in October–November 2015, and here report the second-ever record and isolation from 24 m depth of *Schizocladia ischiensis* E.C.Henry, K.Okuda, and H.Kawai of the poorly known monotypic Schizocladiphyceae, the sister group of the brown algae among the Ochrophyta, enabling novel insights into its habitat and development. The only previous collection of this species had been in 1987 from Ischia, Western Mediterranean [25].

2. Materials and Methods

Diving surveys were conducted off Rhodes between 27 October and 6 November 2015, targeting predominantly the circalittoral zone. The germling emergence method [16] was used to obtain unialgal isolates from substratum samples collected during the dives. Natural substratum was collected by SCUBA divers using 15 mL FALCON tubes previously filled with heat-sterilised sea water, which were not opened before arriving at the sampling sites. Following transport to the laboratory of AFP, the contents of each tube (ca. 5 mL substratum and 10 mL supernatant) were poured into two Petri dishes containing approx. 35 mL half-strength Provasoli-enriched sea water [26] and were incubated at 10–14 °C in dimmed natural daylight. From these, substratum fragments carrying macroalgal germlings were transferred after 40 days into new dishes with culture medium containing 4 mg/L GeO₂ to inhibit diatom growth. Three to four wk later, unialgal isolates were obtained from the dishes by cutting accessible parts of germlings with the sharp edge of a recently torn glass Pasteur pipet and transferred into new dishes containing medium without GeO₂. While other isolates were difficult to identify morphologically and will be described in detail elsewhere after finishing their molecular-assisted identification, isolate RH15-53 resembled microscopically the *Schizocladia ischiensis* strain described previously [25]. The isolate was obtained from substratum sample no. 061115-1, which was collected at Sunwing Beach, Kallithea area (36°24.532' N; 28°14.350' E) at 24 m depth on 06 November 2015 (Figure 1). The site is situated in the infralittoral at the lower end of *Posidonia* beds and above the main coralline zone (Figure 2). The location was chosen for sampling also because fishermen use it as one of their main fishing sites due to its coarse, sandy bottom. It is covered by *Posidonia* beds in patches and has high densities of fish and benthic fauna in general. It is also in the vicinity of the sewage treatment plant of the island.

Strain RH15-53 was deposited in The Kobe University Macroalgal Culture Collection (KU-MACC [27]) and vouchers (permanent microscope mounts) in the herbaria BM [28], PC [29], SAP [30], of the University of Aberdeen (ABDUK) [31] and of the Hellenic Centre for Marine Research

(HCMR) [32]. For re-examination, the previously isolated strain of *Schizocladia* was taken from stock (KU-MACC: KU-333).

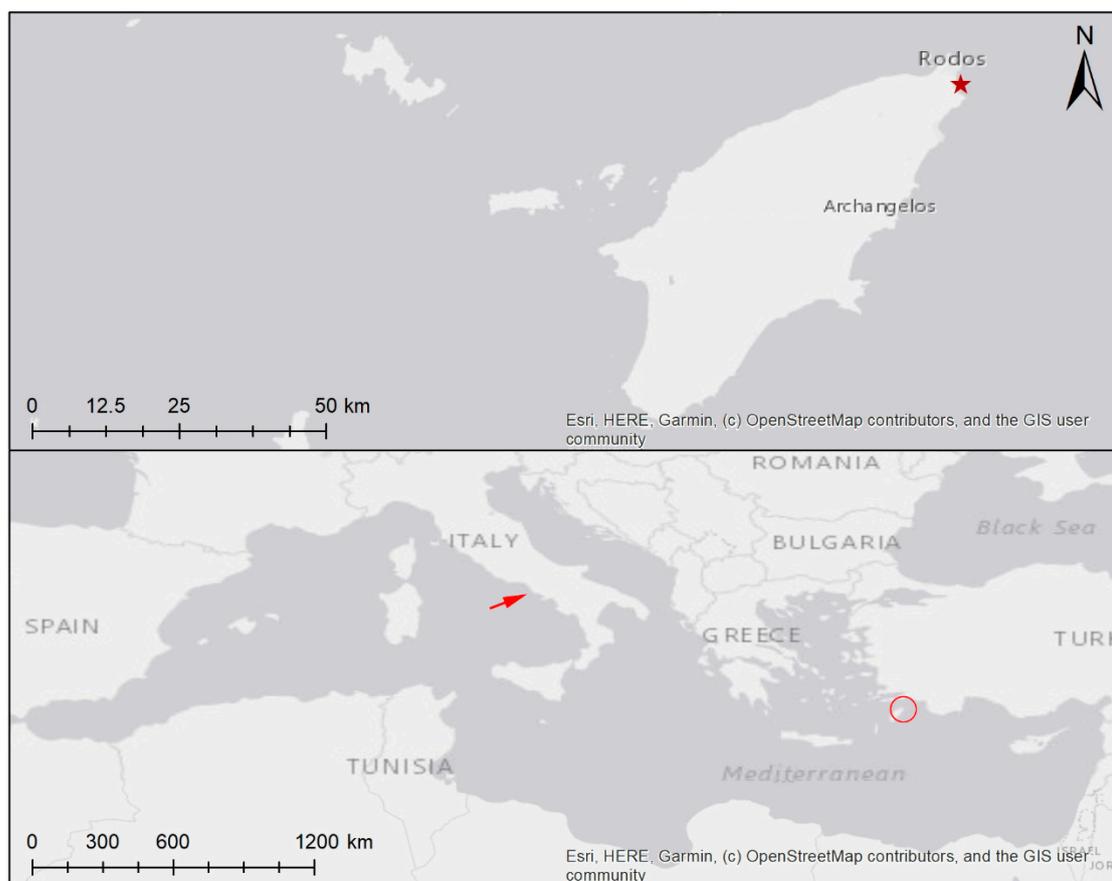


Figure 1. Geographical position of sites. Above: Rhodes Island with asterisk at sampling site. Below: Rhodes (circle) within the Mediterranean. Arrow points to Ischia where the previous isolate of *Schizocladia* was found in 1987.

DNA extractions from strains RH15-53 and KU-333, PCR, and Sanger sequencing were done as described previously [33], targeting the nuclear ribosomal small subunit (18S), the plastid-encoded *rbcLS* spacer (Rubisco spacer) and the genes for *rbcL*, *psaA* and *psbC* by using the primers listed in Table 1. Annealing temperature in the PCRs was 48–55 °C, the number of cycles 35 and the extension temperature 72 °C. The DNA sequences obtained were assembled and aligned manually with Se-AL™ v2.0a11 (Sequencing Alignment Editor Version 2.0 alpha 11 [34]), checked for correctness by inspecting the chromatograms, and compared to published sequences by the Basic Local Alignment Search Tool (BLAST) housed at the United States National Center of Biotechnology Information [35]. The newly determined sequences are deposited in the public database under accessions LC521903-LC521907 (RH15-53), MN994274 (KU-333), and MN996275 (KU-333).

Table 1. Oligonucleotide primers used for PCR amplification and Sanger sequencing.

Primer Name	Marker	Position (Last nt)	Direction	Sequence 5'-3'	Reference
NS1F	nrSSU	35	F	GTAGTCATATGCTTGTCCTC	[36]
NS4R	nrSSU	1108	R	CTTCCGTC AATTCCTTTAAG	[36]
AFP2F	nrSSU	1268	F	AGCTCTTTCTTGATTCTATG	[37]
AFP1R	nrSSU	1796	R	GGTAATGATCCTTCCGCAG	New primer
rbcl77F	rbcL	95	F	TGGGNTAYTGGGATGCTGA	[38]
rbcl952R	rbcL	952	R	CATACGCATCCATTACA	[14], as Ral-R952
rbclP1F	rbcL	965	F	GKGTWATTTGTAARTGGATGCG	[14]
rbcl1273F	rbcL	1273	F	GTGCGACAGCTAACC GTG	[39]
rbcs139R	rbcS	139	R	AGACCCCAT AATCCCAATA	[40]

psaA130F	psaA	130	F	AAACWACWACTTGGATTGGAA	[41]
psaA940R	psaA	940	R	TATGDCCAATWCCCAATT	[41]
psaA870F	psaA	870	F	GGNGGWYTATGGTTAAGTGA	[41]
psaA1760R	psaA	1760	R	CCTCTWCCWGGWCCATCRCAWGG	[41]
psbCF	psbC	37	F	GTGGAAACGCCCTTTAATA	[42]
psbCmidR	psbC	710	R	CCYCCWACDARATCTTCCATATTATC	[42]

For present-day taxonomic and nomenclatural aspects, AlgaeBase [43] was consulted.

3. Results

3.1. Habitat

Approximately 5 mL substratum was collected at 24 m depth. The substratum mainly consisted of coralline algal debris (Figure 2).



Figure 2. Collecting site at 24 m depth, besides a *Posidonia* bed, with coralline algal debris constituting most of the substratum.

3.2. Accompanying Species

From the same substratum sample 061115-1, we isolated 13 other macroalgal germlings, including two green, seven brown, and four red algae, which still await molecular identification and will be treated elsewhere. In cultures from eight other substratum samples, collected from 44 to 52 m depth, we isolated 51 macroalgae (13 green, 32 brown, and 6 red) and did not detect *Schizocladia*.

3.3. Sequences

We generated sequences for the nuclear ribosomal DNA (partial small subunit) and the four plastid markers *rbcL*, Rubisco spacer, *psaA*, and 5'-partial *psbC*, for RH15-53, in total 5237 bp. For comparison with those of the original *Schizocladia* isolate, the DNA for the nuclear ribosomal small subunit and *rbcL* were resequenced, and the Rubisco spacer (82 bp) was newly sequenced in strain KU-333. In SSU, the two strains were identical; in the other markers, they were highly similar but not identical, including cases of nonsynonymous substitutions in *rbcL* and *psaA*. In the Rubisco spacer, the two sequences differed only by a 2 bp indel in the TATA box (Table 2).

Table 2. Results of BLAST searches [35] for sequences of *Schizocladia* from Rhodes. The best hit was in all cases the previous isolate of *S. ischiensis* from Ischia, KU-333 [25,44].

Marker	Length of Examined Sequence (bp)	Identity with Closest Hit (%)	Query Cover (%)	Identity (bp)	Not Identical (bp)	Indels (bp)	Accession of Closest Hit	Comment
SSU-5P	845	100	100	845/845	0	0	MN994274	-
SSU-3P	488	100	100	488/488	0	0	MN994274	-
<i>rbcL</i>	1372	99.42	100	1364/1372	8	0	MN996275	7 synonymous, 1 nonsynonymous Ser > Ala
Rubisco spacer	80	98	100	80/82	2	2	AB085615	An indel of 2 bp length
<i>psaA</i>	1566	99.23	100	1554/1566	12	0	AB908091	11 synonymous, 1 nonsynonymous Ile > Leu
<i>psbC</i> -5P	616	99.84	100	615/616	1	0	AB908094	1 synonymous

3.4. Morphology

Thalli of RH15-53 had the shape of a minute bush of up to ca. 500 μm in height, consisting of cylindrical cells of 15–20 μm in length and 4–5 μm in diameter, which showed subdichotomous or whorled branching. The basal cell of the thallus was wedge-shaped and attached to the petri dish by amorphous material secreted at the lower end of the cell. Plastids were brown and of elongated shape, without pyrenoid (Figure 3a). Reproduction was direct via heterokont motile spores formed by transformation of filament cells (not shown).

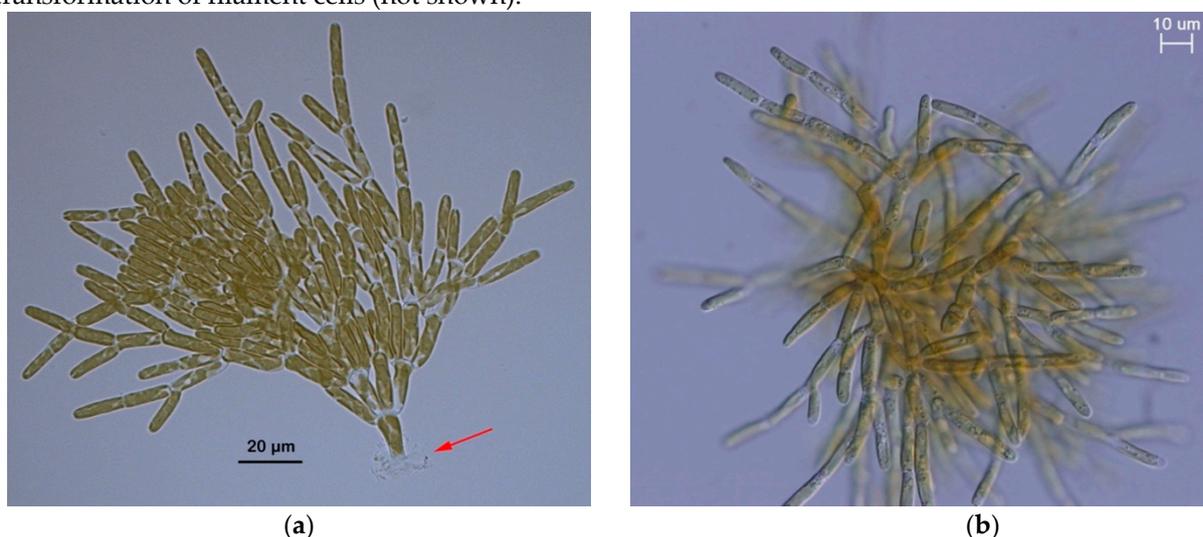


Figure 3. *Schizocladia ischiensis* from laboratory culture. (a) New strain RH15-53 from Rhodes, removed from Petri dish bottom. Original attachment was by amorphous extracellular material (arrow) secreted at lower end of wedge-shaped basal cell (b) Strain KU-333 from Ischia. Note similar dimensions and overall cell morphologies.

4. Discussion

Based on sequences and morphology, isolate RH15-53 was identified as *Schizocladia ischiensis*, forming the second-ever record of this phylogenetically important species. However, the determined sequences for the more variable plastid markers differed slightly from the homologous sequences in the only previous isolate of the species, KU-333 (Table 2) [25]. The similarities to the previous sequences in the plastid-encoded genes were >99%, and 98% in the relatively short noncoding Rubisco spacer. In summary, we interpret the high resemblance of the new sequences as evidence that the Rhodes isolate is conspecific with the previous isolate from Ischia.

Morphologically, our new isolate appeared to differ slightly from the previous isolate because it showed an entirely upright habit consisting of cylindrical cells (Figure 3a). Such a morphology was not described for KU-333 [25], but re-examination showed that a similar habit could also occur in this strain (Figure 3b). Direct reproduction by motile spores in RH15-53 resembled KU-333.

The erect habit of RH15-53 revealed that the thallus of *Schizocladia* lacks any specialised cells that would serve to fix them to the substratum. Instead, attachment of the thallus was by an amorphous,

probably sticky substance. The first cell of the young thallus, which develops from the settled spore, appears to possess polarity because it is upright, and the sticky material is only present at the base of the cell (Figure 3a). This kind of attachment of RH15-53 reminds of species of the Stylonematophyceae (Rhodophyta; [45]) or of colonial benthic diatoms such as *Berkeleya rutilans* (Trentepohl ex Roth) Grunow [46] or *Licmophora flabellata* (Greville) C. Agardh [47]. It is possible that this likely rather weak attachment of *Schizocladia*, Stylonematophyceae, and colonial benthic diatoms is one of the reasons why larger thalli, which would resist to stronger drag forces, have not evolved in these groups. In larger and more complex seaweeds, there is usually early differentiation between the erect part (thallus) and the rhizoid(s), e.g., in Erythropeltiales [48], or stronger attachment is achieved secondarily by specialised structures, e.g., in Discosporangiales [14].

The habitat of the previous isolate of *Schizocladia* was not known because the alga developed in a crude culture of mixed material dredged from different depths [25]. Our isolate was from an infralittoral site (24 m depth), and *Schizocladia* may be a common member of the community of macroalgae occurring in this zone of the Mediterranean sublittoral. We did not find the species in samples from 43 to 52 m depth, which suggests that *Schizocladia* is absent in greater depth. However, such a minute alga can easily be overlooked in culture and overgrown by the other macroalgae, which are generally of larger size. Precise information about the depth distribution of *Schizocladia* would require replicate sampling from the surface to the circalittoral.

In the sublittoral habitat and also in remote locations, phycologists are often constrained by limited time in the field and rudimentary laboratory facilities. Diving work is even more constrained due to limited bottom time and visibility, which is exacerbated with increasing depth. Additionally, in many Mediterranean and tropical coral reef locations, much of the actual macroalgal diversity may not be conspicuous during diving surveys due to the naturally intense grazing activity in such ecosystems [49]. It is not by coincidence that both the original Ischia isolate of *Schizocladia* and ours from Rhodes emerged in the laboratory from cultivated substratum and were only detected by meticulous scrutiny of the algae developing in the dishes. The minute size of the *Schizocladia* thalli resembles that of kelp gametophytes, which are likewise rarely reported from nature [50] although they are abundant [51]. We have recently isolated kelp and *Desmarestia* gametophytes and a minute delesseriacean red alga from incubated substratum collected at sublittoral sites [23,24]. The discovery of *Schizocladia* by again using germling emergence suggests that this technique, albeit labour-intensive, could be the method of choice to reveal the size class of very small multicellular algae from any habitat, particularly the deeper subtidal.

Author Contributions: Conceptualization, A.F.P., F.C.K., and M.D.J.S.; methodology, A.F.P., F.C.K., and M.D.J.S.; software, A.F.P. and T.H.; validation, A.F.P., F.C.K., and H.K.; formal analysis, A.F.P.; investigation, A.R., F.C.K., P.L., E.A., A.O.M.M., M.D.J.S., T.H., H.K., and A.F.P.; resources, F.C.K. and M.D.J.S.; data curation, A.F.P. and T.H.; writing—original draft preparation, A.F.P. and F.C.K.; writing—review and editing, A.F.P. and F.C.K.; visualization, M.D.J.S., F.C.K., and A.F.P.; supervision, A.F.P. and F.C.K.; project administration, F.C.K.; funding acquisition, F.C.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the UK Natural Environment Research Council in support to FCK (program Oceans 2025–WP 4.5 and grants NE/D521522/1 and NE/J023094/1) and the NERC National Facility for Scientific Diving (NFSD/14/02). We would also like to thank the TOTAL Foundation (Project “Brown algal biodiversity and ecology in the Eastern Mediterranean Sea”). This work also received support from AFP from the project IDEALG (France: ANR-10-BTBR-04) and from FCK from the Marine Alliance for Science and Technology for Scotland pooling initiative. MASTS is funded by the Scottish Funding Council (grant reference HR09011) and contributing institutions.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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