

# Taxonomic Reappraisal of *Sphacelaria rigidula* and *S. fusca* (Sphacelariales, Phaeophyceae) Based on Morphology and Molecular Data with Special Reference to *S. didichotoma*

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The taxonomic status of three closely related species of the genus *Sphacelaria*, *S. rigidula* Kützing, *S. fusca* (Hudson) C.F. Gray and *S. didichotoma* Saunders was reassessed on the basis of morphological observations from herbarium specimens and phylogenetic analysis of RUBISCO spacer sequences. *Sphacelaria rigidula* was characterized by having only 2-armed propagules and somewhat slender filaments. Culture experiments revealed that its propagules commonly formed secondary arms, even though they were rarely produced in the field. It is concluded that *S. divaricata* Montagne characterized by dichotomously branched arms in propagules should be synonymized with *S. rigidula*, based on propagule morphology and measurements. *Sphacelaria fusca* clearly be separated from *S. rigidula* by having 2-4 armed propagules. These morphological differences were highly supported by the differences in the RUBISCO spacer sequences. *S. didichotoma* resembles *S. rigidula* in having propagules with two dichotomously branched arms. However, it commonly produces the secondary arms in the field. Additionally, this species has shorter primary arms than *S. rigidula*. Phylogenetic analyses supported the distinction of *S. didichotoma* from *S. rigidula*. The northwestern Pacific plants, previously recorded as *S. divaricata*, were recognized as *S. didichotoma*.

**Key Words:** phylogenetic analysis, propagule morphology, RUBISCO spacer sequences, *Sphacelaria didichotoma*, *S. fusca*, *S. rigidula*

## INTRODUCTION

The brown algal genus *Sphacelaria* subgenus *Propagulifera* is subdivided into three sections on the basis of the shape of propagules: viz. the section *Propagulifera* with fusiform arms and apical hairs, the section *Furcigerae* with mainly cylindrical arms and no apical hairs, and the section *Tribulooides* with tribuliform propagules (Prud'homme van Reine 1982). Of these, the section *Furcigerae* is supposed to include seven to ten species (Prud'homme van Reine 1993; Draisma *et al.* 1998; Keum *et al.* 1999). Species delimitation within this section is mainly based on the distinct shape of the propagules (Kitayama 1994; Draisma *et al.* 1998). The taxonomic status of two widely recorded species, *S. rigidula* Kützing and *S. fusca* (Hudson) C.F. Gray, has

been discussed extensively, and these names have been considered as a being included into one single species having 2-4 armed propagules (Holmes and Batter 1891; Haas-Niekerk 1965; van den Hoek and Flinterman 1968; Russell and Fletcher 1975). However, several authors stated that *S. rigidula* only forms 2-armed propagules (as *S. furcigera*; Sauvageau 1901, 1902; Zimmerman 1925; Irvine 1956; Goodband 1971), whereas *S. fusca* produces 2-3(-4) or only 3-armed propagules (Sauvageau 1901; Setchell and Gardner 1924). Prud'homme van Reine (1982) proposed that it is necessary to separate *S. rigidula* from *S. fusca*, although the taxonomic position of *S. fusca* is still uncertain, because *S. fusca* is different from *S. rigidula* by having thicker erect filaments.

*Sphacelaria divaricata* Montagne was first described on the basis of repeatedly branched 2-armed propagules from Torres Strait (Montagne 1849; Sauvageau 1901) and it has been recorded from the northwestern Pacific and Australia (Lee and Kang 1963; Kitayama 1994; Phillips

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1997; Draisma 1998). Prud'homme van Reine (1982) considered that this species might be a synonym of *S. rigidula*, because a similar branching of the arms frequently occurs in *S. rigidula*. Another species showing repeatedly branched 2-armed propagules, *Sphacelaria didichotoma* Saunders (1898) has been recorded a few times from the northeastern Pacific (Setchell and Gardner 1925; Abbott and Hollenberg 1976; Hansen 1997). However, Kitayama (1994) synonymized this species with *S. divaricata* having similar repeatedly branched 2-armed propagules.

The variability of propagules, including the presence or absence of branching and number of arms, has given rise to much confusion, and thus further investigations are necessary to determine the validity of the four species mentioned above. The aim of the present study was to clarify the taxonomic status of the species based on morphological observations using herbarium specimens and determination of the nucleotide sequence in the RUBISCO spacer region.

## MATERIALS AND METHODS

The specimens examined were loaned from the herbariums coded as AD (State Herbarium of South Australia), AST (Herbarium of the Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China), L (Nationaal Herbarium Nederland, Leiden University branch), MEL (National Herbarium of Victoria, Royal Botanic Gardens, Australia), PC (Muséum National d'Histoire Naturelle, France), SNU (Seoul National University, Korea), TCD (Herbarium, Trinity College, Ireland), TNS (Herbarium, Botany Department, National Science Museum, Japan), and W (Naturhistorisches Museum Wien, Austria). Morphological observations by light microscopy were made from materials preserved in 10% formalin in seawater and from re-soaked (in saturated detergent solution) herbarium specimens. Materials were stained in 1% aqueous aniline-blue solution and mounted in glycerin-seawater solution (1:1) on microscope slides. The slides were photographed with an Olympus BH-2 camera system. The culture was established from apical filaments, which were cultivated in PES medium at 15°C and 12:12 h LD under 15-30  $\mu\text{mol m}^{-2}\text{s}^{-1}$ . The descriptive terminology used by Prud'homme van Reine (1982) and Kitayama (1994) has been followed. Morphological features of herbarium specimens of *Sphacelaria* are provided in Table 1.

Collection information for species used in our molecular investigations is provided in Table 2. The materials were washed with distilled water and dried on filter paper. Genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The concentration of extracted total DNA was determined by comparing the brightness of DNA band on ethidium bromide-stained 0.7% agarose gels. The RUBISCO spacer region was amplified using the primers 1 and 2, which were designed by Stache-Crain *et al.* (1997). PCR amplification was carried out in 50  $\mu\text{L}$  total volume composed of 0.1-20 ng template DNA, 80  $\mu\text{M}$  for each dNTP, 1 unit of Ex Taq polymerase (TaKaRa Bio, Kyoto, Japan), 1x Thermophilic buffer (TaKaRa Bio), 1.5 mM  $\text{MgCl}_2$ , and 10 pmol of each primer. Amplification reactions involved 5 min at 94°C for pre-denaturation, 30 cycles consisting of 30s at 94°C for denaturation, 30s at 48°C for annealing, and 30s at 72°C for extension, with a final extension of 7 min at 72°C, using a GeneAmp PCR Cyclor 2400 (Perkin Elmer, Foster City, CA). The reaction at 4°C after amplification. PCR products were monitored on ethidium bromide-stained 1.0 % agarose gels. For automated sequencing, PCR products were directly sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems). Automated sequencing was used with 377 DNA Sequencing System (Perkin Elmer Applied Biosystems).

Sequences were aligned for phylogenetic analysis using Clustal X (Thompson *et al.* 1999). Phylogenetic trees were inferred from the sequence data using maximum parsimony (MP), maximum likelihood (ML), and neighbor-joining (NJ) methods, as implemented in PAUP\* 4.0 (Swofford 2000). MP analysis was performed using a heuristic search with random sequence addition and Tree-Bisection-Reconnection branch swapping options. All characters were unweighted and unordered, and gaps were treated as missing data. NJ analysis (Saitou and Nei 1987) employed a Kimura (1980) two-parameter model. ML analysis was based on the HKY85 model (Hasegawa *et al.* 1985) with transversions weighted 2 to 1 over transitions and empirical base frequencies. Bootstrap analyses (Felsenstein 1985) were used with 1,000 replicates for all three methods to estimate the stability of tree topologies.

**Table 1.** Comparison of morphological features of the species in the genus *Sphacelaria*

Species	Locality	Specimen	Height	Segment			Propagule			
				Length ( $\mu\text{m}$ )	Width ( $\mu\text{m}$ ) width	Ratio length / width	Length ( $\mu\text{m}$ )	Width of waist ( $\mu\text{m}$ )	Primary arm length ( $\mu\text{m}$ )*	Arm width ( $\mu\text{m}$ )
<i>S. rigidula</i>	Eochungdo, Korea	SNU950629	< 1 cm	52(42-69)	31(25-41)	1.7(1.4-1.9)	326(264-391)	23(18-26)	182(172-213)	21(20-22)
	cultured plant		-	46(40-57)	30(24-38)	1.6(1.2-1.8)	278(220-320)	22(20-25)	386(260-460)	18(15-21)
	Kalbarri, Australia	AD051282	< 5 mm	44(32-55)	34(27-40)	1.3(1.0-1.6)	234(193-331)	24(20-29)	175(164-181)	22(18-26)
	Punarum Pass, Tahiti	L937.117-17	5 mm	45(39-54)	39(33-44)	1.2(1.1-1.3)	265(231-301)	29(23-33)	261(235-286)	24(22-25)
	New Guinea	L934.263-17	5 mm	33(25-39)	38(30-44)	0.9(0.8-0.9)	255(204-293)	30(25-33)	275(248-325)	28(26-30)
<i>S. divaricata</i>	Tanzania	L200346	7 mm	28(21-34)	22(15-26)	1.3(1.0-1.8)	231(186-288)	21(17-24)	161(153-168)	18(16-20)
	Torres Strait, Australia	Type, MA9712	1-2 mm	24-28	20-24	-	160-200	20-30	300-400	-
	Fremantle, Australia	L937.117-15	2-3 mm	46(32-63)	30(23-39)	1.5(1.1-1.8)	162(123-216)	25(22-30)	389(237-493)	24(21-26)
<i>S. fusca</i>	England	Neotype, TCD	3.5 cm	65-96	44-80	1.28-1.73	356-542	36-50	320-540	30-46
	Chaesukgang, Korea	SNU930506	< 2 cm	38(28-49)	42(34-54)	0.9(0.7-1.3)	290(188-290)	29(25-36)	-	28(26-31)
<i>S. didichotoma</i>	Rumoi, Hokkaido Pref., Japan	Kita-61, TNS	< 1.5 cm	44(33-54)	42(35-49)	1.0(0.8-1.4)	301(236-376)	34(26-36)	-	-
	Imagoura, Hyogo Pref., Japan	SNU200409	< 1cm	33(28-43)	39(33-46)	0.9(0.7-1.2)	227(146-307)	41(30-55)	168(103-269)	29(27-32)

\* In field the arm lengths of the propagules of *S. rigidula* varied considerably and rarely branched to form secondary arms. In cultured plant of *S. rigidula* from Eocheongdo, Korea primary arms branched to form secondary arms

**Table 2.** List of species, collection localities, and DNA database accession numbers (GenBank) in this study (Accession numbers for new sequences (this study) are shown in bold)

Species	Collection sites (collectors, collection years)	Accession no.
<i>Sphacelaria didichotoma</i>	Tsuyazaki, Fukuoka, Japan (S. Kawaguchi, 1989)	AJ287949*
<i>S. didichotoma</i>	Imagoura, Hyogo Pref., Japan (Y.-S. Keum, 2000-4-9)	<b>AY898781</b>
<i>S. didichotoma</i>	Maruyama, Awajishima, Hyogo Pref., Japan (Y.-S. Keum, 2000-4-21)	<b>AY898782</b>
<i>S. fusca</i>	Galway, Ireland (W.F. Prud'homme van Reine, 1966-25-1)	AJ287941**
<i>S. fusca</i>	Chaesukgang, Korea (Y.-S. Keum, 1996-5-6)	AJ287944**
<i>S. fusca</i>	Muroran, Hokkaido, Japan (W.F. Prud'homme van Reine, 1993-5-6)	AJ287943**
<i>S. fusca</i>	North America, as <i>S. cirrosa</i> (UTEX)	<b>AY898783</b>
<i>S. rigidula</i>	Cape Verde Islands (H. Pakker, 1991)	AJ287942
<i>S. rigidula</i>	O-ura, Hyogo Pref., Japan (Y.-S. Keum, 2001-6-9)	<b>AY898784</b>
<i>S. rigidula</i>	Haengwon, Cheju Island, Korea (Y.-S. Keum, 1995-5-7)	<b>AY898785</b>
<i>S. rigidula</i>	Eochungdo, Korea (J.H. Oak, 1997-6-12)	<b>AY898786</b>
<i>S. rigidula</i>	Beomsum, Cheju Island, Korea (J.H. Oak, 1998-2-11)	<b>AY898787</b>
<i>S. yamadae</i>	Ogi, Sado Isl., Niigata Pref., Japan (T. Kitayama, 1990)	AJ287950
<i>S. yamadae</i>	Imagoura, Hyogo Pref., Japan (Y.-S. Keum, 2000-5-14)	<b>AY898788</b>
<i>S. californica</i>	Oiso, Awajishima, Hyogo Pref., Japan (W.F. Prud'homme van Reine, 1993)	AJ287951
<i>S. californica</i>	Dokdo Island, Korea (Y.S. Oh, 1995-8-15)	<b>AY898789</b>
<i>S. tribuloides</i>	Sesoko, Okinawa, Japan (W.F. Prud'homme van Reine, 1993)	AJ287947
<i>S. tribuloides</i>	Jan Thiel, Curacao (A.H. van Engelen, 1999)	AJ287948
<i>S. cirrosa</i>	Fife Ness, Scotland (S.G.A. Draisma, 1977)	AJ287946

\* in Draisma 2002: as *S. divaricata*

\*\* in Draisma 2002: as *S. rigidula*

## RESULTS

### Morphological observations

#### *Sphacelaria rigidula* Kützing 1843, p. 292

##### Figs 1-12

**Holotype:** Red Sea, on *Cystoseira triquetra*, Schimper s.n. (L 937.117-229; Isotype, W 19682).

**Synonyms:** *Sphacelaria furcigera* Kützing 1855, p. 27, Karak Island, Persian Gulf (L 937.714-72). *Sphacelaria divaricata* Montagne 1849, p. 62, Torres Strait, Australia (Holotype, MA 9712, PC [Herb. Montagne], Isotype, TCD).

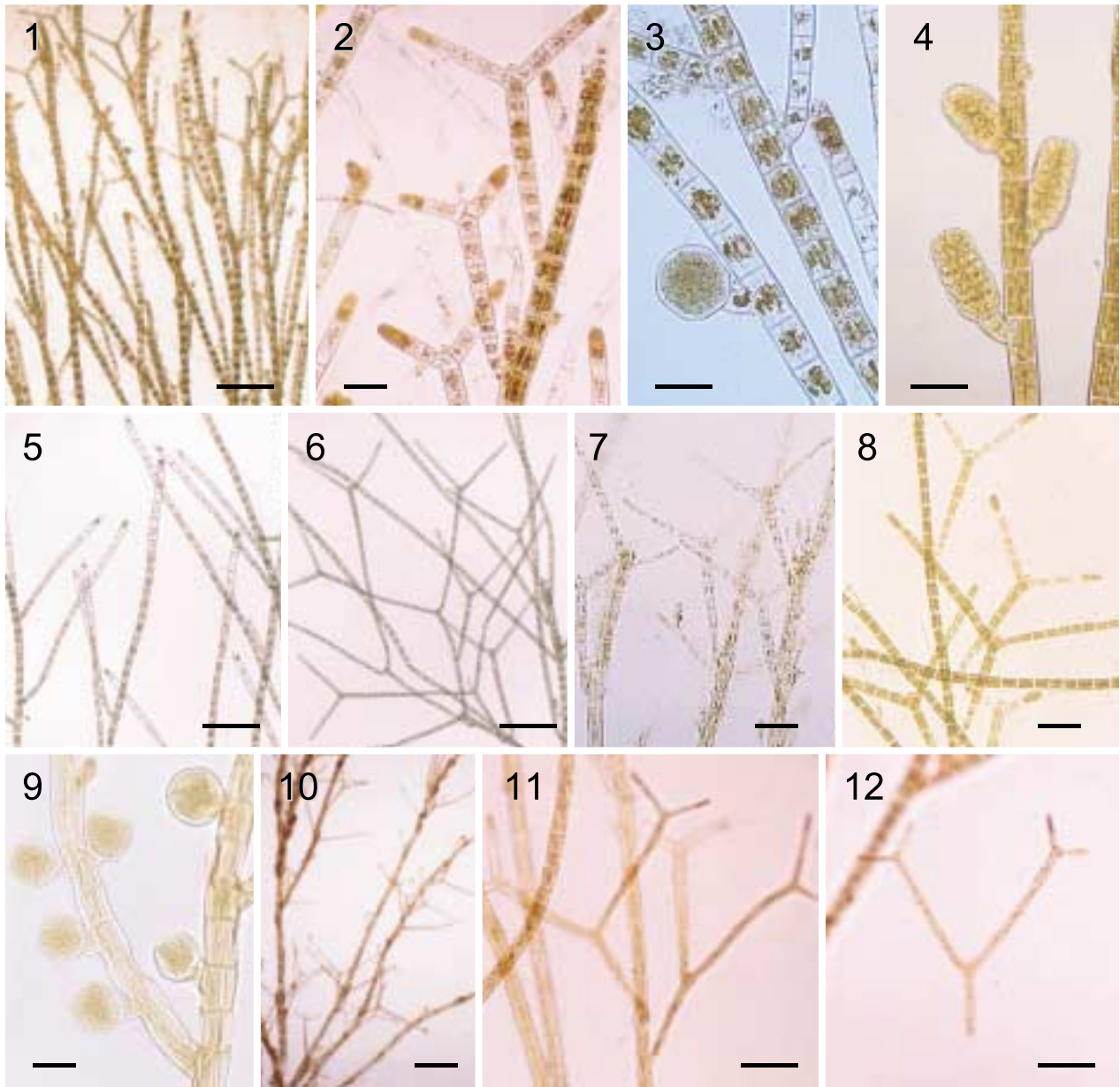
**Distribution:** warm temperate to tropical areas (Prud'homme van Reine 1982)

**Specimens examined:** KOREA. Eochungdo, 29 VI 1995, J.H. Oak (SNU950629). Haengwon, Cheju Island, 7 VI 1995, Y.-S. Keum (SNU950607). Beomsum, Cheju Island, 11 II 1998, J. H. Oak (SNU980211). JAPAN. O-ura, Hyogo Pref., 9 VI 2000, Y.-S. Keum (SNU20609). TAHITI. Punarum Pass, 2 VII 1922, Setchell and Rarks (L 937.117-17). NEW GUINEA ISLANDS. No specific locality and collector, 25 VIII 1899 (L 934.263-17). INDONESIA. No locality, date and collector (L 937.117-215). AUSTRALIA. WA. Jacques Pt, Kalbarri, 15 IX 1979, H.B.S. Womersley,

(AD 51282). Point Valliant, Two People Bay, mid eulittoral pools, 30. viii. 1979, H.B.S. Womersley (L 471206). No specific locality and collector, 26 IV 1875 (W 19656). NEW ZEALAND. Stewart Island, 13 VI 1945, no collector (L 947.192-1). DAHOMEY. Cotonov, lower eulittoral zone, 26 III 1971, D.M. John and G.W. Lawson (L, no number). TANZANIA. Mafia-Wiene Island, 7 IX 1967, no collector (L 200346).

***S. divaricata*.** AUSTRALIA. Torres Strait, on *Sargassum* sp., no date and collector, MA 9712 (Holotype, PC [Herb. Montagne], Isotype, TCD). Kanala, on *Turbinaria*, no collector and date (herb. Le Jolis, MEL 501112). Fremantle, W.F. Prud'homme van Reine, no date (L 937.117-15). Rockingham, no collector and date (L 937.117-18); Rockingham, no date, Demel s.n. (W 19651). Port Denison, no collector and date (W 19658). OCEANIA. New Caledonia, Kanala, no collector and date (L 937.117-19).

Thalli erect, 0.5-1.0 cm high, brown to olive brown, tufts to cushioned, epiphytic. Holdfasts monostromatic, discoid and stoloniferous. Erect filaments straight and cylindrical with indeterminate irregular lateral branches (Fig. 1). Segments 21-69  $\mu\text{m}$  long, 15-44  $\mu\text{m}$  broad, 0.8-1.9 in L/B ratio at main axes, divided radially by 2-3(-4) longitudinal walls. Peripheral cells rectangular, 3-10  $\mu\text{m}$



**Figs 1-9.** *Sphacelaria rigidula* Kützing. **Figs 1-6.** Plant from Eochungdo, Korea. **Fig. 1.** Filaments bearing propagules. **Fig. 2.** Two-armed propagules. **Fig. 3.** Unilocular zoidangia. **Fig. 4.** Plurilocular zoidangia. **Fig. 5.** Filaments of cultured material. **Fig. 6.** Repeatedly branched two-armed propagules. **Fig. 7.** Two-armed propagules from Tahiti (L937. 117-17). **Fig. 8.** Two-armed propagules from Australian plants (AD51282). **Fig. 9.** Unilocular zoidangia (AD51282). **Figs 10-12.** *Sphacelaria divaricata* from Australia. **Fig. 10.** Filaments bearing propagules (W19651). **Fig. 11.** Propagules with dichotomously branched arms (L937. 117-19). **Fig. 12.** A propagule with dichotomously branched arms (L. 937. 117-15) (Scale bars: Figs 1, 5, 6, 10; 200  $\mu\text{m}$ ; Figs 2, 7, 8, 11, 12; 100  $\mu\text{m}$ ; Figs 3, 4, 9; 50  $\mu\text{m}$ ).

in diameter and scarcely divided by secondary transverse walls. Phaeophycean hairs 8-12  $\mu\text{m}$  in diameter, directly on apical cells or by oblique division of apical cells (Fig. 2). Propagules frequently in upper and middle filaments, consisting of two straight, cylindrical and symmetrical primary arms and a stalk in field-collected specimens (Fig 2). Stalks of propagules 186-391  $\mu\text{m}$  long, 17-33  $\mu\text{m}$  broad at waist. Primary arms

153-325  $\mu\text{m}$  long, 16-30  $\mu\text{m}$  broad, but these lengths varied considerably in populations (Table 1). In culture, two primary arms branched to form secondary arms, 260-460  $\mu\text{m}$  long (Fig. 6). Unilocular zoidangia produced in middle to lower parts of filaments, dark brown, spherical, 29-55  $\mu\text{m}$  in diameter, with a one-celled pedicel (Figs 3, 9). Plurilocular zoidangia occur in middle to lower parts of filaments, 45-65  $\mu\text{m}$  long, 25-40  $\mu\text{m}$



**Figs 13-20.** *Sphacelaria fusca* from Korea. **Fig. 13.** Filaments bearing propagules. **Figs 14, 15.** 2-3 armed propagules irregularly arranged on filaments. **Fig. 16.** A four armed propagule. **Fig. 17.** Unilocular zoidangia in lower part of filaments. **Fig. 18.** Spherical unilocular zoidangia. **Fig. 19.** Macroplurilocular zoidangia. **Fig. 20.** Microplurilocular zoidangia (Scale bars: Figs 13, 17; 200  $\mu\text{m}$ ; Figs 14-16; 100  $\mu\text{m}$ ; Figs 18-20; 50  $\mu\text{m}$ ).

broad, with a one-celled stalk (Fig. 4). Loculi 3-7  $\mu\text{m}$  in diameter. No differentiation into micro- and macroplurilocular zoidangia.

**The type and herbarium specimens:** The type was small, about 2-4 mm high, and has a few filaments with indeterminate laterals. Only two propagules with two primary arms were found. Morphological characteristics of additional herbarium specimens of *S. rigidula* are provided in Table 1. These materials had slender filaments and propagules with always only two primary arms (Figs 7, 8).

**The type and herbarium specimens of *S. divaricata*:** The type specimen is up to 1-2 mm high, epiphytic on *Sargassum* sp., and has erect filaments with indeterminate, irregularly branched laterals. Segments of filaments are 24-28  $\mu\text{m}$  long, 20-24  $\mu\text{m}$  broad. Propagules are composed of two slender primary arms and a stalk with 20-30  $\mu\text{m}$  waist-width and 160-200  $\mu\text{m}$  long. The two primary arms are 2-3 times branched to form additional arms, and in those cases the primary arms

grown up to 300-400  $\mu\text{m}$  in length. No data are available on unilocular and plurilocular zoidangia.

Additional herbarium specimens deposited in AD, MEL and L collected from Australia and tentatively recorded as *S. rigidula* or *S. furcigera* corresponded well to the type of *S. divaricata*. They have quite slender filaments and propagules with two arms, which are repeatedly dichotomously branched to form additional arms (Figs 10, 11, 12).

***Sphacelaria fusca* (Hudson) S.F. Gray 1821, p. 333.**

**Figs 13-22**

**Type:** Sidmouth, England, June 1827, *Griffiths s.n.* (Neotype, TCD).

**Basionym:** *Conferva fusca* Hudson 1762.

**Synonyms:** *Sphacelaria subfusca* Setchell et Gardner 1924, p. 13, f. 25, Redondo, southern California, xi 1908 (Holotype specimen probably lost, is now fig. 25 of the publication).

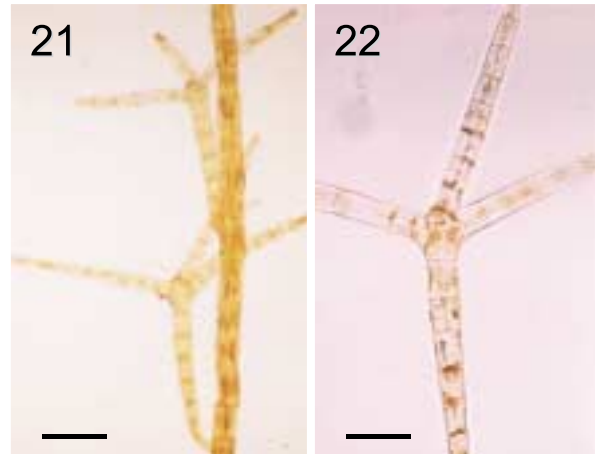
**Distribution:** warm temperate to tropical areas

(Prud'homme van Reine 1982)

**Specimens examined:** ENGLAND. Sidmouth, VI 1827, *Griffiths s.n.*, (Neotype, TCD). Exmouth, 1851, *Gulson* (TCD). FRANCE. Saint Malo, Port Royal, 9 VI 1872, *Sauvageau* (Herb. Thuret, PC). KOREA. Chaesukgang, 6 V 1993, Y.-S. Keum (SNU930506). Jeoncheon, 6 VIII 1993, Y.-S. Keum (SNU930806). As *S. rigidula*. JAPAN. Rumoi, Hokkaido Pref., 2 VIII 1989, T. Kitayama, (Kita-61, TNS). Iwata, Aomori Pref., 7 IV 1991, T. Kitayama (Kita-162, TNS). Uratomi, Hyogo Pref., 7 IV 1990, T. Kitayama (Kita-104, TNS). CHINA. Qingdao, 30 VI 1994, Y.S. Oh (SNU940630). Guangdong, no date and collector (AST 55-1679). Guangdong, no date and collector (AST 55-1860).

Thalli erect, dark brown to olive brown, tufts, complanate or cushioned, epiphytic on *Sargassum thunbergii*, *Corallina* spp., *Laurencia* spp., and reaching up to 3 cm high. Holdfasts monostromatic to polystromatic, discoid and stoloniferous. Erect filaments cylindrical, with indeterminate irregularly branched laterals (Fig. 13). Segments 26-46  $\mu\text{m}$  long, 32-54  $\mu\text{m}$  broad, 0.65-1.10 in L/B at main axes with 2-6 longitudinal walls. Peripheral cells rectangular 5-13  $\mu\text{m}$  in diameter and secondary transverse walls often formed. Phaeophyceyan hairs directly on apical cells or formed by oblique division of apical cells, 8-14  $\mu\text{m}$  in diameter. Propagules irregularly but densely scattered in upper to middle filaments (Fig. 13) consisting of straight, cylindrical 2-3(-4) primary arms, a straight stalk and a lenticular apical cell (Figs 14, 15, 16). Stalks of propagules 188-330  $\mu\text{m}$  long, 23-35  $\mu\text{m}$  broad at waist. In culture, primary arms often branched to form the secondary arms. Unilocular zoidangia produced in middle to lower parts of filaments (Fig. 17), dark brown, spherical, 38-63  $\mu\text{m}$  in diameter with a one-celled pedicel (Fig. 18). Plurilocular zoidangia formed on middle to lower parts of filaments. Macro- and microplurilocular zoidangia on different plants. Macroplurilocular zoidangia, with large locules (5-10  $\mu\text{m}$  in diameter), dark brown, ellipsoid, 45-102  $\mu\text{m}$  long, 53-79  $\mu\text{m}$  broad, 0.85-1.48 in L/B (Fig. 19). Microplurilocular zoidangia, with small locules (2-6  $\mu\text{m}$  in diameter), yellowish brown, ellipsoid, 75-131  $\mu\text{m}$  long, 41-70  $\mu\text{m}$  broad, 1.39-2.57 in L/B (Fig. 20).

**The type specimen:** The plant is about 3 cm high and consists of irregularly and scarcely branched indeterminate filaments and a few 3-armed propagules. Segments of filaments are 65-96  $\mu\text{m}$  long and 44-80  $\mu\text{m}$  broad. Stalks of propagules are 356-542  $\mu\text{m}$  long, 36-50



**Figs 21, 22.** Neotype of *Sphacelaria fusca* (Sidmouth, England, TCD). Propagules with 3 arms (Scale bars: Fig. 21, 100  $\mu\text{m}$ ; Fig. 22, 50  $\mu\text{m}$ ).

$\mu\text{m}$  broad at waist; primary arms are 320-540  $\mu\text{m}$  long (Figs 21, 22)

### *Sphacelaria didichotoma* Saunders 1898, p. 158

#### Figs 23-30

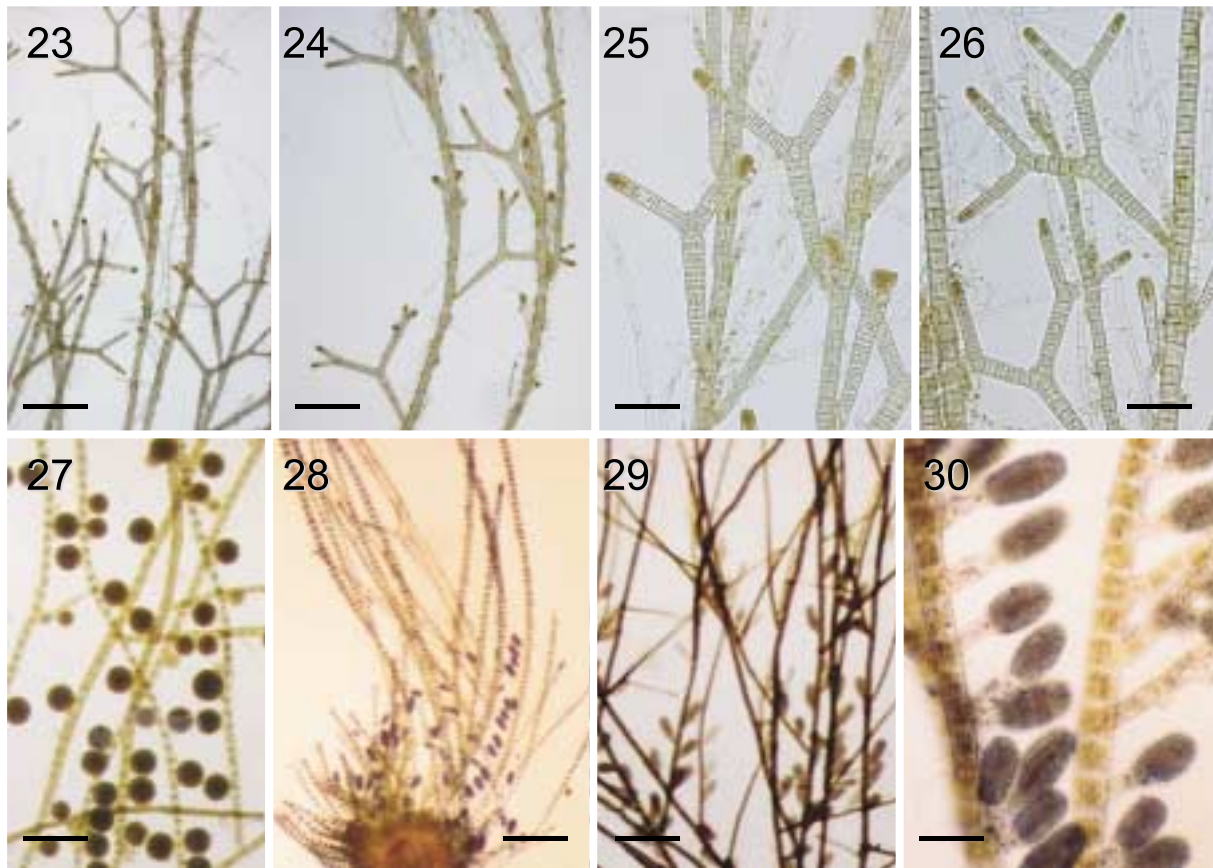
**Type:** Carmel Bay, California, Specimen probably lost, Holotype Saunders 1898, pl. XXVII, fig. 1)

**Synonyms:** *Sphacelaria divaricata* f. *japonica* Takamatsu 1943, p. 165-166, f. 6, pl. 10. *Sphacelaria hizikiae* Ohta et Noda 1984, p. 33-38, f. 1. *Sphacelaria prostrata* Takamatsu 1943, p. 160, pl. 9, f. 1, 2.

**Distribution:** Pacific Ocean; Korea (Lee and Kang 1986), Japan (Kitayama 1994) and California (Setchell and Gardner 1925, Abbott and Hollenberg 1976).

**Specimens examined:** KOREA. Eodal, 29 V 1993, Y.-S. Keum (SNU930529). Anin, 21 X 1993, Y.-S. Keum (SNU931021). Seongsan, Cheju Island, 21 III 1994, Y.-S. Keum (SNU940321). As *S. divaricata*. JAPAN. Tappi, Aomori Pref., 24 V 1989, T. Kitayama 42 (TNS). Fukaura, Aomori Pref., 25 V 1989, T. Kitayama 37 (TNS). Tsuyazaki, Fukuoka Pref., 2 IV 1989, S. Kawaguchi (TNS, Kita-37). Shimoda, Shizuoka Pref. on *Hizikia fusiformis* in the middle to lower intertidal zone, 20 V 1990, J. Tanaka (L 993.356-480). Imagoura, Hyogo Pref., 9 IV 2000, Y.-S. Keum (SNU200409). Maruyama, Awajishima, Hyogo Pref., 21 IV 2000, Y.-S. Keum (SNU200421). CHINA. Guangdong, no collector and date (AST 56-2970).

Thalli erect up to 1 cm high, dark brown to olive brown, tufts or cushioned, epiphytic. Holdfasts monostromatic to polystromatic, discoid and stoloniferous. Erect filaments cylindrical, with



**Figs 23-30.** *Sphacelaria didichotoma* from Japan. **Figs 23, 24.** Mature propagules with dichotomously branched arms (Imagoura, Hyogo Pref.). **Fig. 25.** Young propagules with 2 arms (Maruyama, Hyogo Pref.). **Fig. 26.** Propagules with dichotomously branched arms (Maruyama, Hyogo Pref.). **Fig. 27.** Unilocular zoidangia. **Fig. 28.** Plurilocular zoidangia in lower part of erect filaments. **Fig. 29.** Microplurilocular zoidangia. **Fig. 30.** Macroplurilocular zoidangia (Scale bars: Figs 23, 24, 28, 200  $\mu\text{m}$ ; Figs 25-27, 29, 30, 100  $\mu\text{m}$ ).

indeterminate irregularly branched laterals. Segments, 28-43  $\mu\text{m}$  long, 33-46  $\mu\text{m}$  broad, 0.7-1.2 in L/B at main axes, divided by 2-6 longitudinal walls. Peripheral cells rectangular 5-13  $\mu\text{m}$  in diameter, with occasional secondary transverse walls. Phaeophycean hairs 10-16  $\mu\text{m}$  in diameter, directly on apical cells or formed by oblique division of apical cells. Propagules frequent in upper to middle filaments (Figs 23, 24), consisting of two dichotomously branched arms, a stalk and a lenticular apical cell (Figs 25, 26). Stalks of propagules 146-307  $\mu\text{m}$  long, 30-55  $\mu\text{m}$  broad at waist, tapering slightly and gradually to the base. Two primary arms curved slightly backward and branched to form secondary arms, 103-269  $\mu\text{m}$  long. Unilocular zoidangia produced in middle to lower parts of filaments (Fig. 27), dark brown, spherical, 30-60  $\mu\text{m}$  in diameter, with a one-celled pedicel. Plurilocular zoidangia formed on middle to lower parts of filaments (Fig. 28). Macro- and microplurilocular zoidangia on different plants. Macroplurilocular zoidangia, with large locules (4-10  $\mu\text{m}$  in diameter), dark

brown, ellipsoid, 40-100  $\mu\text{m}$  long, 50-75  $\mu\text{m}$  broad, 0.7-1.3 in L/B (Fig. 30). Microplurilocular zoidangia, with small locules (2-5  $\mu\text{m}$  in diameter), yellowish brown, ellipsoid, 45-70  $\mu\text{m}$  long, 30-35  $\mu\text{m}$  broad, 1.2-2.3 in L/B (Fig. 29).

### Molecular study

The sequences of RUBISCO spacer ranged for 7 species of *Sphacelaria* from 234 to 276 base pairs. The lengths of the RUBISCO spacer were 261-264 bp in *Sphacelaria rigidula*, 252 bp in *S. fusca* and 234-238 bp in *S. didichotoma*. The sequence of RUBISCO spacer appeared to be conserved at the specific level in *S. fusca* and *S. rigidula*, except for one specimen of *S. rigidula*. The sequence of *S. rigidula* for three Korean specimens and one Japanese specimen were identical, but considerably differed from the Cape Verde Islands specimen (5.4%). The three Japanese specimens in *S. didichotoma* were showed a relatively high divergence of 0.4-2.1%. The interspecific variations of sequences among *S. rigidula*, *S.*



*fusca* and *S. didichotoma* ranged from 10% to 20.3%. Sequence divergence for seven *Sphacelaria* species included in this study ranged from 6.3 to 31.2%. The sequence divergences between *S. cirrosa* (section *Propagulifera*) and the other studied members in the sections *Furcigerae* and *Tribuloides* of *Sphacelaria* were considerably high (in the range of 21.1-31.2%). The sequence divergences between at the one side *S. californica* or *S. tribuloides* in the section *Tribuloides* and at the other side members of the section *Furcigerae* were relatively similar to those among members in the section *Furcigerae*.

A total of 289 nucleotide characters were in the present study available for phylogenetic analyses. Among them, 28 variable characters were phylogenetically uninformative and 86 characters were informative. Parsimony analysis resulted in a single most parsimonious tree (tree length = 176 steps, CI=0.81, RI=0.91) (Fig. 31). NJ and ML analyses generated trees of which the topology was identical with the most parsimonious tree. *S. cirrosa* was used as an outgroup based on a phylogenetic analysis of *rbcl* sequences (Draisma 2002).

Our phylogenetic tree showed that the three species, *S. rigidula*, *S. fusca* and *S. didichotoma* were clearly recognized. However, within *S. fusca* and the Korean and Japanese specimens of *S. rigidula*, relationships among strains could not be determined further due to identical sequences. *Sphacelaria fusca* and *S. didichotoma* formed a clade together with *S. californica*. The latter was basal within this clade, which was strongly supported by high bootstrap values (99%). *Sphacelaria rigidula* and *S. yamadae* constituted other clade (100%). *S. tribuloides* was placed as a sister group to these two clades, therefore *S. californica* and *S. tribuloides* placed in the section *Tribuloides* were separated each other, implying that the sections *Furcigerae* and *Tribuloides* of the subgenus *Propagulifera* do not constitute monophyletic groups.

## DISCUSSION

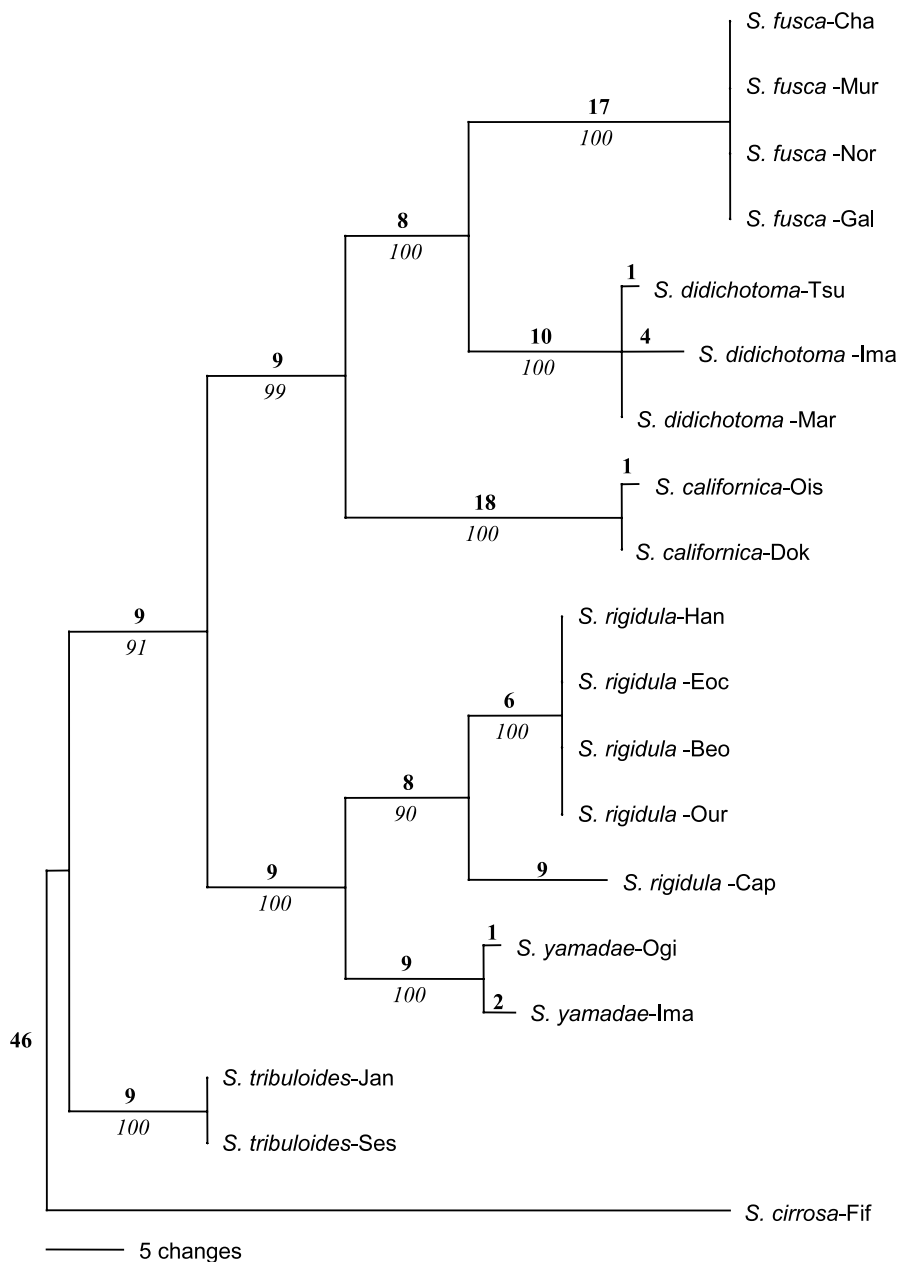
*Sphacelaria furcigera* was first described by Kützing (1855) from Karak Island, Persian Gulf. on the basis of plant with the 2-armed propagules. Hudson (1762) described *Conferva fusca* from England, and later S.F. Gray (1821) transferred it to the genus *Sphacelaria* and mentioned on 3-armed propagules. However, a type specimen was not designated. Harvey (1841) recorded *S. fusca* from Sidmouth and St. Michaels Mount, England.

Prud'homme van Reine (1982) confirmed that Hudson's specimens were lost and designated Harvey's specimen collected from Sidmouth as a neotype.

Many phycologists have considered that there are no morphological differences between *S. fusca* and *S. furcigera* (Holmes and Batters 1891; De Haas-Niekerk 1965; Van den Hoek and Flinterman 1968; Russell and Fletcher 1975). De Haas-Niekerk (1965) mentioned that there is no reason to separate two species on the basis of the similarity in the production of two-armed propagules by both species and synonymized *S. furcigera* with *S. fusca*. Van den Hoek and Flinterman (1968) accepted De Haas-Niekerk's opinion, but preferred use the name *S. furcigera*. However, some authors noted that *S. furcigera* shows always only two arms in propagules, while *S. fusca* has two to four arms. The proportion of the number of arms in propagules in *S. fusca*, on the other hand, is quite variable (Sauvageau 1902; Zimmermann 1925; Goodband 1971).

*Sphacelaria rigidula* was established by Kützing (1843) from the Red Sea, but at that time the presence of propagules was not mentioned. Prud'homme van Reine (1982) found 2-armed propagules from the type specimen of *S. rigidula* and synonymized *S. furcigera* with *S. rigidula*. He regarded *S. rigidula* as abundant and widely dispersed plants with 2-4 arms as well as with only 2 arms in propagules. *S. fusca*, however, was considered by him as a rare species, consistent with the neotype showing thicker filaments and the absence of rhizoids and stolons, although it has 2-3 armed propagules. When Kitayama (1994) monographed Japanese *Sphacelaria* species, he recorded that the type specimen of *S. rigidula* has just a few 2-armed propagules (fig. 25A), but all of 53 propagules in the type of *S. furcigera* are only 2-armed (fig. 25B). Following Prud'homme van Reine's opinion, however, he tentatively considered the Japanese plants with 2-4 armed propagules as *S. rigidula*, but mentioned that the taxonomic entity of these Japanese plants needed further discussion.

In our opinion, however, *Sphacelaria rigidula* is clearly distinguished from *S. fusca* by having only 2-armed propagules. We found that the type of *S. furcigera* has 2-armed propagules as well as the type of *S. rigidula*. Thus the type materials of both taxa collected in the tropical Red Sea contained only 2-armed propagules. On the basis of the examination of herbarium specimens and our collections from Japan and Korea, we propose to use *S. rigidula* for the species occurring widely in warm



**Fig. 31.** The single most parsimonious tree inferred from the RUBISCO spacer sequences. For codes see table 2. The consistency index was 0.81, and the retention index was 0.91. Numbers above branches represent nucleotide substitutions. Numbers below branches indicate bootstrap values

temperate and tropical areas. *S. fusca* is characterized by 2-4 armed propagules and is also widely distributed throughout the world. In addition, *S. rigidula* has usually slightly slender erect filaments (15-44  $\mu\text{m}$  vs. 32-54  $\mu\text{m}$ ), have a smaller waist width (17-33  $\mu\text{m}$  vs. 23-35  $\mu\text{m}$ ), and also a smaller primary arm width (16-30  $\mu\text{m}$  vs. 26-31  $\mu\text{m}$ ) than *S. fusca*. Therefore, the Japanese materials examined by Kitayama (1994) should be assigned to *S. fusca*. As well as these morphological features, *S. rigidula* was clearly separated from *S. fusca* in the sequence divergence of 17.5-18.9 %. It formed a clade with,

showing a close genetic distance to *S. yamadae* (6.3-8.2%). These two species are commonly collected on *Sargassum* spp. from subtidal areas, whereas the other species occur both in intertidal and subtidal areas. The Korean and Japanese specimens of *S. rigidula* from various localities showed no sequence variation. However, the specimen from Cape Verde Islands showed highly different sequences (5.4%), may due to geographical distance. In *S. fusca*, the RUBISCO spacer sequence data did not resolve at population level, although various Atlantic and Pacific populations are included.

The neotype of *S. fusca* from Sidmouth is somewhat atypical, compared with other collections, in regards to the width of its erect filaments (almost twice as wide as those of most collections). Prud'homme van Reine (1982) also mentioned that the rare plants described as *S. fusca* differ from common plants described as *S. rigidula* by a larger diameter in erect filaments (35-80  $\mu\text{m}$ ) and in the stalks of the propagules (29-47  $\mu\text{m}$ ). However, he added that the differences in diameter of the erect filaments in the distal parts are inconspicuous and the absence of rhizoid is probably due to lack of sufficient materials. Additionally, we could not detect specimens in other herbarium collections that were in size agreement with the neotype. The herbarium collections of *S. fusca* show propagules with 2-3(4) arms and all had a similar (smaller) size in erect filaments. Thus, it is considered that the size difference of the neotype just represents a deviation from the typical form of *S. fusca*.

Womersley (1987) mentioned that the Australian plants of *S. fusca* show opposite and alternate branches with broad branching and prominent axes. However, these features are in accordance with the defining characters of *S. cirrosa* showing the laterals distichously and pinnately branched and the propagules with apical hairs and 3 fusiform or cylindrical arms curved backward, although occasionally propagules without apical hairs occurred. The examinations of Womersley's specimens from AD showed that some specimens have the laterals branched alternately and pinnately as well as propagules with constrictions at the base and apical hairs, thus they are considered as *S. cirrosa*.

Setchell and Gardner (1924) described *S. subfusca* from specimens with 2-3 armed propagules collected in California, and mentioned that it is distinguished from *S. fusca* in having propagules which are often bifurcate, without tapering of stalk or of arms. However, Prud'homme van Reine (1982) suggested that *S. subfusca* is closely related to *S. fusca* (as *S. rigidula*), and Kitayama (1994) has treated *S. subfusca* in the synonymy of *S. rigidula*. Although the type specimen of *S. subfusca* was probably lost, the original description and figures are consistent with *S. fusca* on the basis of propagule morphology. Additionally, the proportion of the number of arms in *S. fusca* is variable, and the stalk and arms may slightly taper or do not at all. We therefore concluded that *S. subfusca* should be reduced to synonymy with *S. fusca*.

Since the description of *S. divaricata* by Montagne (1849), that species has been recorded from the

northwestern Pacific, including Japan (Kitayama 1994), Korea (Kang 1966; Lee and Kang 1986) and China (Draisma *et al.* 1998). Sauvageau (1901) stated that it is characterized by repeatedly branched two-armed propagules, as exceptionally also occurring in *S. rigidula* (as *S. furcigera*). Prud'homme van Reine (1982), however, stated that the criteria to distinguish *S. divaricata* by Sauvageau (1901) are irrelevant, because the branching of arms of the propagules occurs frequently in *S. fusca* (as *S. rigidula*). When Kitayama (1994) recorded that Japanese *Sphacelaria* plants show dichotomously branched arms in the propagules, he investigated the type specimen of *S. divaricata*, and mentioned that the primary arms of the propagules in this type are 200 - 490  $\mu\text{m}$  in length, whereas the Japanese plants designated under that name are just up to 250  $\mu\text{m}$ . Nevertheless he identified the Japanese plants as *S. divaricata* and synonymized *S. didichotoma* with *S. divaricata* sharing the same arm morphology in their propagules.

Examination of the type of *S. divaricata* showed slender filaments (20-24  $\mu\text{m}$ ) and long dimensions (300-400  $\mu\text{m}$ ) in the primary arm length. In culture, we found that the arms of the propagules in *S. rigidula* commonly branched to form additional secondary arms, and that their primary arms reached up to 260-460  $\mu\text{m}$  in length. In general, in the species of the section *Furcigerae* the primary arms are able to branch again to form additional arms in propagules. In the Korean plants and other herbarium specimens of *S. rigidula*, however, secondary arms are rarely found in the field materials. Therefore, the type of *S. divaricata* is considered as an old plant producing secondary arms in its propagules before they detach. Additionally, the type and herbarium specimens of *S. divaricata* show slender filaments similar to those of *S. rigidula*. According to these characteristics and dimensions, it is reasonable that *S. divaricata* should be regarded as a synonym of *S. rigidula*.

The Japanese plants that have previously been recorded as *S. divaricata* form dichotomously branched propagules, showing shorter primary arms (103-269  $\mu\text{m}$ ) than the type of *S. divaricata* and the cultured plants of *S. rigidula*. These dimensions are in accordance with the dimensions of Kitayama's plants. Abbott and Hollenberg (1976) recorded that in *S. didichotoma* from California the primary arms were 100-200  $\mu\text{m}$  in length. Moreover, *S. didichotoma* in the field usually produces secondary arms from two primary arms. Therefore, the northwestern Pacific plants are assigned to *S. didichotoma*. The type specimen of *S. didichotoma*, however, was probably lost,

but the northwestern Pacific plants are well agreement with the original features and descriptions. Thus fig.1 (pl. XXVII) of Saunders (1898) has to be considered as the holotype. Kitayama (1994) synonymized two Japanese species, *S. prostrata* Takamatsu (1943) and *S. hizikiae* Ohta and Noda (1984) with *S. divaricata*. Therefore, these two species should subsequently be considered as synonyms of *S. didichotoma*. Recently, Keum *et al.* (2001) proposed that *S. divaricata* f. *japonica* belongs to *S. divaricata*, because it has dichotomously divided two-armed and asymmetrical propagules with the secondary arms branched after the fourth secondary segment of the primary arms as seen in *S. divaricata*. Thus, it is also a synonym of *S. didichotoma*. In our phylogenetic tree, the three Japanese specimens of *S. didichotoma* formed a clade with *S. fusca*, but showed a higher sequence divergence (0.4-2.1%) than *S. fusca* and *S. rigidula* (except for the Cape Verde Islands population).

Phylogenetic relationship among the species in *Sphacelaria* subgenus *Propagulifera* based on RUBISCO spacer sequences is thus well resolved. However, *S. californica* and *S. tribuloides* do not belong to the same monophyletic group. Thus it appears that both the section *Furcigerae* and the section *Tribuloides* are not monophyletic. However, only a few strains of the two sections were investigated up to now. In previous molecular phylogenetic study, the two sections, *Furcigerae* and *Tribuloides* were also found not to be monophyletic in the tree inferred from the *rbcL* and RUBISCO spacer sequences data (Draisma 2002). Draisma (2002) mentioned that the propagule morphology, used as a traditional morphological character to separate the sections in subgenus *Propagulifera*, is not phylogenetically informative within the subgenus *Propagulifera*. Additionally, he recommended that the sections *Furcigerae* and *Tribuloides* either no longer be recognized or that they be merged into one section. Consequently, the subgenus *Propagulifera* still needs taxonomic revision. However, at present there are insufficient morphological and molecular data to support any useful taxonomic treatment.

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