Protist

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Global Diversity and Biogeography of *Skeletonema* Species (Bacillariophyta)

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Abstract

Recent studies have shown that the cosmopolitan diatom *Skeletonema costatum* sensu lato is composed of several morphologically and genetically distinct species. To assess whether the separate species have a cosmopolitan distribution, we analysed 184 strains from marine and estuarine sites worldwide. We identified the strains using light and electron microscopy, and we sequenced the hyper-variable region of nuclear LSU rDNA. All recently described species were genetically distinct, and all but two were morphologically distinct. Variability was found for the only ultrastructural character used to distinguish *Skeletonema dohrnii* and *S. marinoi*, which cannot be identified based on morphology alone. Furthermore, multiple genetically distinct taxa, which may represent cryptic species, were found within the *S. menzelii* and *S. tropicum* clades. We found that all currently recognized species of *Skeletonema* are widespread, however, gaps seem to occur in their geographical ranges. For example, some species are found in both the northern and southern temperate latitudes whereas other species appear to have only subtropical to tropical ranges. *Skeletonema pseudocostatum* and *S. grethae* seem to have more restricted geographical ranges because the former was not found along American coasts and the latter was encountered only in US waters. A taxonomic update is provided for *Skeletonema* strains currently available in several

Abbreviations: AC, Algobank Caen; CCAP, Culture Collection of Algae and Protozoa; CCMP, Provasoli-Guillard National Center for Culture of Marine Phytoplankton; CS, culture collection of CSIRO; CSIRO, Commonwealth Scientific and Industrial Research Organisation; CTCC, Cape Town Culture Collection at the Marine and Coastal Management; GoN, Gulf of Naples; LM, light microscopy; LSU rDNA, gene region coding for nuclear large sub-unit ribosomal RNA; MBA, Marine Biological Association; NIES, National Institute for Environmental Studies; PLY, Plymouth Algal Culture Collection; SCXM, Culture Center of Xiamen University; SEM, scanning electron microscopy; TEM, transmission electron microscopy, SZN, Stazione Zoologica A Dohm, Naples

culture collections, which could aid reinterpretation of results obtained in comparative studies using these strains.

Key words: biogeography; cryptic species; distribution; LSU rDNA; phytoplankton; *Skeletonema costatum*.

Introduction

The diversity and distribution of free-living microbes have intrigued scientists for centuries (e.g., Baas Becking 1934; Costa 1838; Fenchel 2003; Finlay 2002). High growth rates and minute dimensions can foster huge populations that in turn provide large numbers of individuals for mass dispersal (Finlay 2002). Consequently, microbial organisms are believed to occur wherever the environment permits ("everything is everywhere, but, the environment selects;" Baas Becking 1934; de Wit and Bouvier 2006). Moreover, mass dispersal and cosmopolitanism may prohibit allopatric speciation, and hence, diversity of these organisms is believed to be low (Finlay et al. 1996, 1999; Fenchel 2005; Finlay and Fenchel 2004). The alternative view is that at least some microbial species show endemism, as demonstrated in freshwater habitats (Telford et al. 2006, 2007; Theriot et al. 2006; Vyverman et al. 2007).

Perception of protist distribution may be affected by conservative criteria used to distinguish species. In this case, apparent cosmopolitanism would be a consequence of overly inclusive taxonomic units. Several recent studies revealed that phenotype-based species each consisted of two or more genetically distinct clades with identical or subtly different morphologies (e.g., Amato et al. 2007; Behnke et al. 2004; Beszteri et al. 2005; Darling et al. 2004; de Vargas et al. 1999, 2002; John et al. 2003; Koch and Ekelund 2005; Lowe et al. 2005; Lundholm et al. 2002, 2003; Mann 1999; Mann et al. 2004; Montresor et al. 2003; Nanney et al. 1998; Orsini et al. 2004; Sáez et al. 2003; Sarno et al. 2005, 2007; Šlapeta et al. 2006). The genetic differences were confirmed as biologically relevant when reproductive barriers were found among the distinct units (Amato et al. 2007; Behnke et al. 2004; Mann 1999; Vanormelingen et al. 2007). Also in several cases, ecological differences were detected among the genetically distinct strains (de Vargas et al. 1999, 2002; Rodriguez et al. 2005).

The centric diatom *Skeletonema costatum* sensu lato (s. l.) (Greville) Cleve is another example of a microorganism where gross morphology masks genetic diversity. *Skeletonema* species are common phytoplankters, especially in coastal estuarine and marine environments where they often form dense blooms (e.g., Castillo et al. 1995; Cleve 1900; Cloern et al. 1985; Estrada et al. 1985; Hasle 1973; Huang et al. 2007; Karentz and Smayda 1984). *Skeletonema costatum* s. l. was considered morphologically plastic (Hasle 1973), genotypically diverse (Gallagher 1980, 1982, 1994), physiologically versatile (Brand 1984; Paasche 1975; Rijstenbil et al. 1989a, b), and was found worldwide except in the Antarctic Ocean. Three other species, *S. menzelii*, *S. subsalsum* and *S. tropicum*, were generally recognized. However, *S. costatum* s. l. was recently shown to consist of several distinct entities. First, *S. pseudocostatum* was described as a separate species (Medlin et al. 1991). Next, two morphologically distinct species, *S. costatum* sensu stricto (s. s.) and *S. grevillei* were found in the type material (Zingone et al. 2005). Thereafter, five additional species (*S. ardens, S. dohrnii, S. grethae, S. japonicum* and *S. marinoi*) were described, in addition to those already described (Sarno et al. 2005, 2007). All of these species were delineated using morphological traits (e.g., shape of the external fultoportulae processes) and SSU/LSU rDNA sequence differences. In some cases, regional distribution patterns suggested ecological differences that supported species distinctions (Castillo et al. 1995; Sarno et al. 2005, 2007; Chen et al. 2007). Reproductive isolation was not tested because *Skeletonema* is homothallic according to observations by Migita (1967).

In this study, we explore whether currently recognized *Skeletonema* species are as widespread as the historic *S. costatum* s. l. We examine numerous strains from different geographical locations. Strains are identified using morphological information (Chen et al. 2007; Sarno et al. 2005, 2007; Zingone et al. 2005) and the D1—D4 region of the nuclear LSU rDNA. We also address if species distinctness is retained when specimens are obtained from many different regions.

Table 1. List of strains used in this study, with their names, strain ID's, geographical origin, and GenBank numbers of their partial LSU rDNA sequences

Strain ID	Geographical origin	Ocean	Collection dd/mm/yy	GenBank LSU rDNA
Skeletonema ardens Sarno et Zingone (Sarno et al. 2007)				
CCMP794	Singapore	NEI	−/12/73	DQ396492
SCXM13	Xiamen Harbour, P.R. China	NWP	30/08/05	As above
CS-347	Gulf of Carpentaria, Australia	SWP	−/−/91	DQ396493
CS-348	Gulf of Carpentaria, Australia	SWP	−/−/91	DQ396494
Skeletonema costatum (*Greville*) Cleve emend. Zingone and Sarno (Zingone et al. 2005)				
SZN B202[a]	Indian River Lagoon, FL, USA	NWA	28/01/03	DQ396489
SZN B203[a]	Indian River Lagoon, FL, USA	NWA	28/01/03	DQ396490
SZN B206[a]	Montevideo, Uruguay	SWA	29/03/04	As below
SZN B207[a]	Montevideo, Uruguay	SWA	29/03/04	DQ396491
SZN B211[a]	Montevideo, Uruguay	SWA	29/03/04	As above
SZN B212[a]	Montevideo, Uruguay	SWA	29/03/04	As above
B[a]	Museu, Lagoa dos Patos, Brazil	SWA	29/10/04	As above
C[a]	Museu, Lagoa dos Patos, Brazil	SWA	29/10/04	As above
SK-DH[b]	Donghai Sea, P.R. China	NWP	05/07/03	DQ234258
SK-XM[b]	Xiamen, Taiwan Strait, P.R. China	NWP	22/09/04	DQ234260
Skeletonema dohrnii Sarno et Kooistra (Sarno et al. 2005)				
SZN B104 CCMP2479	Marechiara, GoN, Italy	MED	04/02/02	AJ633537
SZN B105 CCMP2480	Marechiara, GoN, Italy	MED	12/02/02	AJ633538
SZN B191 CCMP2481	Marechiara, GoN, Italy	MED	05/02/04	As above
SZN B224[a]	Marsdiep, The Netherlands	NEA	−/04/04	As above
PLY612[a]	English Channel	NEA	02/06/03	As above
SK-FQ[b]	Qingdao, Yellow Sea, P.R. China	NWP	16/05/04	DQ234259
SK-BH[b]	Bohai Sea, P.R. China	NWP	04/04/05	DQ234262
NIES-16	Harima Nada, Seto Inland Sea, Japan	NWP	−/02/83	DQ396508
NIES-17	Harima Nada, Seto Inland Sea, Japan	NWP	−/02/83	As above
NIES-323	Kishiwada, Osaka Bay, Japan	NWP	−/01/85	As above
NIES-324	Kobe, Osaka Bay, Japan	NWP	−/07/85	As above
CS-408	Swan Estuary, WA, Australia	SEI	Autumn 96	As above
CCMP1715	Kasitsna Bay, AK, USA	NEP	−/04/95	As above
CCMP779 CS-79	Bass Strait, Australia	SWP	?	DQ396510
CCMP789	06.4666N 54.9833W, Chain Cruise 48, Suriname	NWA	05/06/65	As above
CCMP782	Auke Bay, AK, USA	NEP	09/04/79	DQ396509
CCMP785	Saanich Inlet, BC, Canada	NEP	−/−/76	As above
NIES-223	Shodo Isl. Kagawa, Japan	NWP	−/07/79	DQ396507
SCDT01[a]	Wenzhou, P.R. China (From resting stage)	NWP	26/09/04	DQ396511
Skeletonema grethae Zingone et Sarno (Sarno et al. 2005)				
SZN B190[a]	Indian River Lagoon, FL, USA	NWA	05/01/03	AJ633523
SZN B192 CCMP2508	Indian River Lagoon, FL, USA	NWA	05/01/03	As above
SZN B194 CCMP2509	Indian River Lagoon, FL, USA	NWA	28/01/03	As above
SZN B195[a]	Indian River Lagoon, FL, USA	NWA	28/01/03	As above
SZN B197[a]	Indian River Lagoon, FL, USA	NWA	05/01/03	As above
SZN B199[a]	Indian River Lagoon, FL, USA	NWA	05/01/03	As above
CCMP775	28.9020N 89.4853W, off Mississippi delta, Gulf of Mexico, LA, USA	NWA	06/02/80	As above
CCMP776	Freeport, TX, USA	NWA	−/−/72	DQ396503
CCMP780	Eel Pond, Woods Hole, MS, USA	NWA	−/−/74	AJ633522

Table 1. (*continued*)

Strain ID	Geographical origin	Ocean	Collection dd/mm/yy	GenBank LSU rDNA
CCMP1804	Narragansett Bay, RI, USA	NWA	01/08/95	As above
CCAP1077/3	Narragansett Bay, RI, USA	NWA	—/10/86	AJ633521
NOAA-A1[a]	NOAA dock, Beaufort, NC, USA	NWA	12-17/05/04	As above
NOAA-B1[a]	NOAA dock, Beaufort, NC, USA	NWA	12-17/05/04	As above
NOAA-B2 CCMP2801	NOAA dock, Beaufort, NC, USA	NWA	12-17/05/04	As above
NOAA-C1[a]	NOAA dock, Beaufort, NC, USA	NWA	12-17/05/04	As above
NOAA-A1[a]	NOAA dock, Beaufort, NC, USA	NWA	12-17/05/04	DQ396504
NOAA-D1[a]	NOAA dock, Beaufort, NC, USA	NWA	12-17/05/04	As above
NOAA-D3[a]	NOAA dock, Beaufort, NC, USA	NWA	12-17/05/04	As above
Skeletonema grevillei Sarno et Zingone (Zingone et al. 2005)				
CCMP1685	23.6500N 58.7000E, Arabian Sea, Muscat, Oman	NWI	Autumn 93	DQ396495
SCXM09	Xiamen Harbour, P.R. China	NWP	26/07/05	DQ396496
Skeletonema japonicum Zingone et Sarno (Sarno et al. 2005)				
SZN B149 CCMP2506	Hiroshima Bay, Seto Inland Sea, Japan	NWP	01/10/02	AJ633524
CCMP784	Uncle Sam Bank, Baja California, Mexico	NEP	—/10/65	As above
CCMP1281	South California Bight, near Santa Cruz Island; CA, USA	NEP	Winter '90	As above
CCMP1283	South California Bight, near Santa Cruz Island, CA, USA	NEP	Winter '90	As above
CS1104024 (Korea)	Changmong-ni, Koje-Do I., Korea 128°39′35″E, 35°00′40″N	NWP	—/02/04	As above
CS2104025 (Korea)	Koje-Do I., Korea 128°28′10″E, 34°51′12″N	NWP	—/04/04	As above
CTCC-32	Lambert's Bay, South Africa	SEA	—/03/03	As above
Hen-A6[c]	Hentiesbaai, Namibia	SEA	30/11/06	As above
Nar-2W[a]	Narragansett Bay, RI, USA	NWA	07/12/04	As above
Nar-3[a]	Narragansett Bay, RI, USA	NWA	07/12/04	As above
Nar-4W[a]	Narragansett Bay, RI, USA	NWA	07/12/04	As above
Chile[a]	Bahía Coliumo, Dichato, Chile	SEP	—/—/05	As above
CS-76[d]	Port Hacking, NSW, Australia	SWP	19/07/78	As above
Skeletonema marinoi Sarno et Zingone (Sarno et al. 2005)				
SZN B118 CCMP2497	Northern Adriatic Sea, Italy	MED	22/02/02	AJ633530
SZN B119 CCMP2501	Northern Adriatic Sea, Italy	MED	22/02/02	AJ633531
SZN B120 CCMP2502	Northern Adriatic Sea, Italy	MED	22/02/02	AJ633532
SZN B121 CCMP2503	Northern Adriatic Sea, Italy	MED	09/03/01	AJ633533
SZN B146 CCMP2504	Northern Adriatic Sea, Italy	MED	09/03/01	AJ633534
SZN B147 CCMP2505	Northern Adriatic Sea, Italy	MED	22/02/02	AJ633535
SZN B189 CCMP2507	Hong Kong, P.R. China	NWP	Summer 01	AJ633529
SZN B205[a]	Marsdiep, The Netherlands	NEA	—/04/04	As above
SZN B216[a]	Marsdiep, The Netherlands	NEA	—/04/04	As above
SZN B217[a]	Marsdiep, The Netherlands	NEA	—/04/04	As above
SZN B218[a]	Marsdiep, The Netherlands	NEA	—/04/04	As above
7A3[a]	Umeå, Sweden	NEA	11/06/03	As above
14A4[a]	Umeå, Sweden	NEA	11/06/03	As above
PLY106	English Channel, UK	NEA	04/07/50	As above

Table 1. (*continued*)

Strain ID	Geographical origin	Ocean	Collection dd/ mm/yy	GenBank LSU rDNA
PLY582	English Channel, UK	NEA	04/07/50	As above
CCMP781	Fladenground, North Sea, UK	NEA	—/04/76	As above
Helgoland[a]	Helgoland, Germany	NEA	Unknown	As above
RF-B[a]	Raunefjord, Bergen, Norway	NEA	18/01/06	As above
RF-C[a]	Raunefjord, Bergen, Norway	NEA	14/02/06	As above
RF-F[a]	Raunefjord, Bergen, Norway	NEA	21/02/06	As above
RF-G[a]	Raunefjord, Bergen, Norway	NEA	13/06/06	As above
RF-H[a]	Raunefjord, Bergen, Norway	NEA	22/06/06	As above
CCMP791	Unknown		Unknown	DQ396505
CCMP1009	39.2000N 69.3333W, continental slope, (USA)	NWA	—/—/77	AJ633536
CCMP1016	08S 80W, Peru (upwelling)	SEP	—/—/76	As CCMP791
CCMP1223	Unknown		Unknown	DQ396506
CCMP1224	Unknown		Unknown	As CCMP781
CCMP1225	Unknown		Unknown	As above
CCMP1226	Unknown		Unknown	As above
CCMP1332 CCAP1077/5 NEPCC782 CS-181	Milford Harbor, Long Island Sound, CT, USA	NWA	—/—/56	As above
AC-174	Luc-sur-Mer, Normandy, France	NEA	—/—/87	As above
AC-623	Salt-water lake near Bouin, Pais de la Loire, France	NEA	Unknown	As above
CAN A1[b]	Georgia Strait, BC, Canada	NEP	—/03/03	AY699024
CAN B2[b]	Georgia Strait, BC, Canada	NEP	—/03/03	AY699025
CAN D2[b]	Georgia Strait, BC, Canada	NEP	—/03/03	AY699026
CAN E2[b]	Georgia Strait, BC, Canada	NEP	—/03/03	AY699027
CAN G1[b]	Georgia Strait, BC, Canada	NEP	—/03/03	AY699028
CAN J1[b]	Georgia Strait, BC, Canada	NEP	—/03/03	AY699029
CAN K2[b]	Georgia Strait, BC, Canada	NEP	—/03/03	AY699030
PORT A3[b]	Lisbon, Portugal	NEA	—/03/03	AY699044
PORT C3[b]	Lisbon, Portugal	NEA	—/03/03	AY699045
PORT D2[b]	Lisbon, Portugal	NEA	—/03/03	AY699046
PORT F3[b]	Lisbon, Portugal	NEA	—/03/03	AY699048
PORT G3[b]	Lisbon, Portugal	NEA	—/03/03	AY699047
KAL C3[b]	Kalmar, Swedish East coast	NEA	—/03/03	AY699031
KAL D2[b]	Kalmar, Swedish East coast	NEA	—/03/03	AY699032
KAL E1[b]	Kalmar, Swedish East coast	NEA	—/03/03	AY699033
KAL H1[b]	Kalmar, Swedish East coast	NEA	—/03/03	AY699034
KAL H3[b]	Kalmar, Swedish East coast	NEA	—/03/03	AY699035
KAL N2[b]	Kalmar, Swedish East coast	NEA	—/03/03	AY699036
KAL N3[b]	Kalmar, Swedish East coast	NEA	—/03/03	AY699037
KF L3[b]	Koljöfjord, Swedish West coast	NEA	—/02/03	AY699039
KF N2[b]	Koljöfjord, Swedish West coast	NEA	—/02/03	AY699038
KF S1[b]	Koljöfjord, Swedish West coast	NEA	—/03/03	AY699041
KF S2[b]	Koljöfjord, Swedish West coast	NEA	—/03/03	AY699042
KF R2[b]	Koljöfjord, Swedish West coast	NEA	—/03/03	AY699040
KF Y2[b]	Koljöfjord, Swedish West coast	NEA	—/03/03	AY699043
Skeletonema menzelii Guillard, Carpenter et Reim (Guillard et al. 1974)				
SZN B82[a]	Marechiara, GoN, Italy	MED	18/12/01	AJ633526
SZN B83[a]	Marechiara, GoN, Italy	MED	18/12/01	AJ633525
SZN B138[a]	Marechiara, GoN, Italy	MED	18/12/01	As above
SZN B219 CCMP2799	Marsdiep, The Netherlands	NWA	—/04/04	As above
SZN B220[a]	Montevideo, Uruguay	SWA	29/03/04	As above
CCMP786	Unknown		Unknown	As above
Naos13[a]	Isla Naos, Gulf of Panama, Panama	NEP	20/01/06	EF423400
CCMP787	32.1667N 64.5000W, Sargasso Sea	NWA	22/05/59	AJ633527

Table 1. (*continued*)

Strain ID	Geographical origin	Ocean	Collection dd/mm/yy	GenBank LSU rDNA
CCMP790	Chase Creek, Dennis, MA, USA	NWA	—/09/79	AJ633528
CCMP792	Patuxent River, Benedict, MD, USA	NWA	Summer 85	As above
CCMP793	Perch Pond, Falmouth, MA, USA	NWA	18/07/82	As above
CCMP795	Wellfleet Harbor, Wellfleet, MA, USA	NWA	—/03/82	As above
Skeletonema pseudocostatum Medlin emend. Zingone et Sarno ([Sarno et al. 2005](#))				
SZN B77 CCMP2472	Marechiara, GoN, Italy	MED	29/05/01	AJ633507
SZN B78 CCMP2473	Marechiara, GoN, Italy	MED	18/12/01	AJ633508
SZN B79 CCMP2474	Marechiara, GoN, Italy	MED	08/01/02	AJ633509
SZN B80 CCMP2475	Marechiara, GoN, Italy	MED	21/01/02	AJ633510
SZN B81 CCMP2476	Marechiara, GoN, Italy	MED	29/01/02	AJ633511
SZN B139 CCMP2477	Marechiara, GoN, Italy	MED	12/03/02	AJ633512
SZN B140 CCMP2478	Marechiara, GoN, Italy	MED	20/05/02	AJ633513
Villefranche[a]	Villefranche-sur-Mer, France	MED	15/05/05	As above
CCAP1077/7	Alexandria, Egypt	MED	—/—/83	AJ633514
FKM 2[b]	Koljöfjord, Swedish West coast (from resting stage)	NEA	Sediment	EF423399
CS-167	South Australia	SWP	07/01/83	As above
CS-252	Moreton Bay, Cleveland, Qld, Australia	SWP	10/01/89	As above
CS-367	Dunalley, Tasmania, Australia	SWP	—/—/97	As above
SCXM01[a]	Xiamen, P.R. China	NWP	early/03/04	As above
D1[c]	Summerstrand, Port Elizabeth, South Africa	SWI	05/12/06	As above
D2[a]	Summerstrand, Port Elizabeth, South Africa	SWI	05/12/06	As above
CLA1.D6[c]	Fish Hoek, Valsbaai, South Africa	SEA	17/01/07	As above
CLA3.A2[a]	Natures Valley, South Africa	SWI	26/01/07	As above
CLA3.B1[a]	Natures Valley, South Africa	SWI	26/01/07	As above
CLA3.B6[c]	Natures Valley, South Africa	SWI	26/01/07	As above
Skeletonema cf. *pseudocostatum*[e]				
A[a]	Casino Beach, Lagoa dos Patos, Brazil (as *S.* cf. *pseudocostatum*)	SWA	06/10/05	DQ396497
Skeletonema subsalsum (Cleve) Bethge ([Bethge 1928](#))				
CCAP1077/8	Lower Lough Erne, Co. Fermanagh, Northern Ireland, UK	NEA	Unknown	AJ633539
Skeletonema tropicum Cleve ([Cleve 1900](#))				
SZN B141 CCMP2800	Marechiara, GoN, Italy	MED	03/10/02	AJ633515
SZN B142[a]	Marechiara, GoN, Italy	MED	12/11/02	AJ633516
SZN B143 CCMP2803	Marechiara, GoN, Italy	MED	12/11/02	AJ633517
SZN B144[a]	Marechiara, GoN, Italy	MED	12/11/02	AJ633518
SZN B145[a]	Marechiara, GoN, Italy	MED	12/11/02	AJ633519
SZN M98[a]	Marechiara, GoN, Italy	MED	14/04/04	DQ396499
SZN M99 CCMP2798	Marechiara, GoN, Italy	MED	14/04/04	As above
SZN B162[a]	Marechiara, GoN, Italy	MED	Unknown	As above
SZN B164[a]	Marechiara, GoN, Italy	MED	Unknown	As above
SZN MC593[a]	Marechiara, GoN, Italy	MED	23/09/03	As above

Table 1. (*continued*)

Strain ID	Geographical origin	Ocean	Collection dd/ mm/yy	GenBank LSU rDNA
CCMP778	Tortuguero River, Costa Rica	NWA	—/09/68	DQ396498
CCMP788	Galveston Channel, TX, USA	NWA	—/—/73	AJ633520
CS-604[f]	Mackay, Queensld, Australia	SWP	22/03/96	As above
CS-605[f]	Mackay, Queensld, Australia	SWP	22/03/96	As above
SCXM03	Xiamen Harbour, P.R. China	NWP	04/07/05	As SZN M98
SCXM07	Xiamen Harbour, P.R. China	NWP	08/07/05	As above
SCND03[a]	Wenzhou, P.R. China (from resting stage)	NWP	10/03/05	As above
SK-HH[b]	Jiaozhou Bay, Yellow Sea, P.R. China	NWP	10/10/03	DQ234261
SZN B205[a]	Montevideo, Uruguay	SWA	29/03/04	DQ396501
SZN B208[a]	Montevideo, Uruguay	SWA	29/03/04	DQ396502
SZN B209[a]	Montevideo, Uruguay	SWA	29/03/04	DQ400578
SZN B210 CCMP2802	Montevideo, Uruguay	SWA	29/03/04	As SZN B208
SZN B213[a]	Piriápolis, Uruguay	SWA	29/03/04	As above
SZN B214[a]	Piriápolis, Uruguay	SWA	29/03/04	As above
SZN MM[a]	Montevideo, Uruguay	SWA	29/03/04	As above
D[a]	Museu, Lagoa dos Patos, Brazil	SWA	15/02/05	DQ396500
CCMP2070	Perlas Islands, Gulf of Panama, Panama	NEP	01/03/97	DQ400577

Ocean regions see Figure 3. "—" in the collection date signifies date unknown; Ocean abbreviations are explained in Figure 3. Abbreviation within strain ID indicates culture collection at which strain is available (see list of abbreviations).

[a]No longer available.
[b]Sequences from Godhe et al. (2006), from Anna Godhe, or from Chen et al. (2007).
[c]Available from Stuart Sym, stuart.sym@wits.ac.za.
[d]Reported as *S. pseudocostatum* in Medlin et al. (1991). Previously listed as such at the CSIRO culture collection.
[e]Ultrastructurally conforms to *S. pseudocostatum.*
[f]Not commercially available from CSIRO culture collection.

Results

Species Diversity and Sequence Diversity

Morphological and molecular identification of 184 investigated strains showed the presence of *S. ardens* (4 strains), *S. costatum* s. s. (10), *S. dohrnii* (19), *S. grethae* (18), *S. grevillei* (2), *S. japonicum* (13), *S. marinoi* (57), *S. menzelii* (12), *S. pseudocostatum* (21), *S. subsalsum* (1) and *S. tropicum* (27) (Table 1). Strains of *S. japonicum* often showed wider fultoportular process tips than described previously (Sarno et al. 2005). Despite this difference, which will be detailed elsewhere, *S. japonicum* was identified by chloroplast number and frustule ultrastructure (Sarno et al. 2005). Strains morphologically identified as *S. tropicum* and *S. menzelii* formed at least two and three distinct genetic groups, respectively, in LSU analyses (Fig. 1). Strains identified as *S. pseudocostatum* had highly similar ultrastruc-

tural features: with 1—2 chloroplasts, the typical 1:1 (rarely 1:2) relationships among processes of sibling cells, narrow fultoportular tips, and tubular fultoportular bases on terminal cells. The LSU rDNA sequences were virtually identical except for a Lagoa dos Patos (Brazil) strain, which is hereafter referred to as *S.* cf. *pseudocostatum*.

Nineteen strains were identified as *S. dohrnii* and 57 as *S. marinoi* based on molecular data alone. In the original descriptions (Sarno et al. 2005), cingular band ultrastructure was the only morphological difference observed between the two species although the authors already reported variability for this character in *S. dohrnii*. However, in the present study bands typical for *S. dohrnii* were also observed in some strains genetically belonging to *S. marinoi*.

Phylogenetic relationships were well resolved and most clades had bootstrap support (bs)
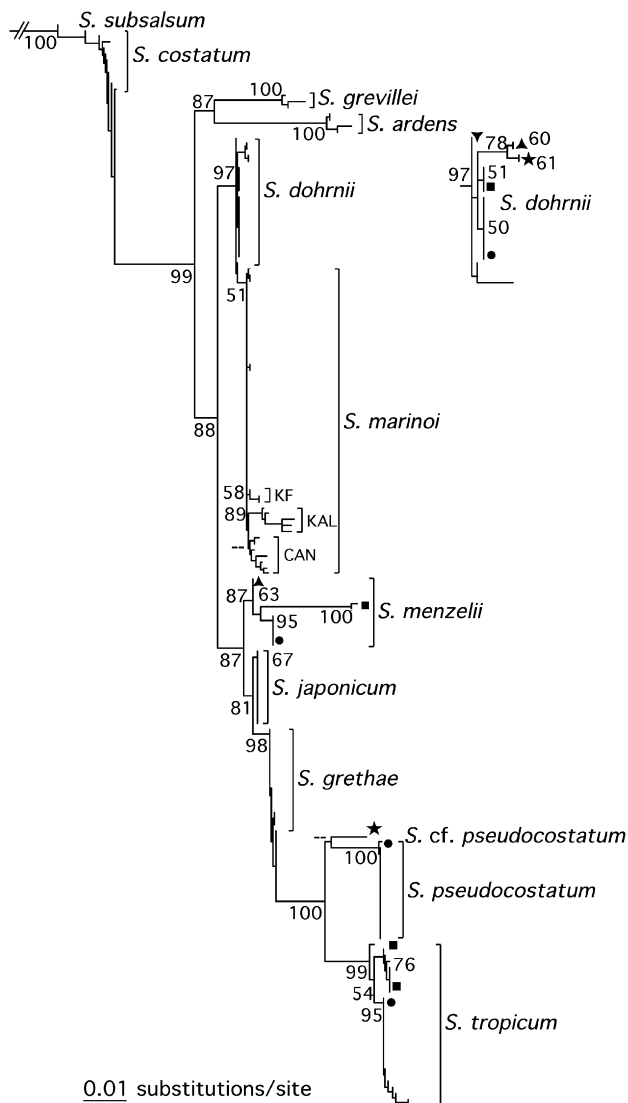
Figure 1. Neighbour joining phylogram inferred from partial LSU rDNA sequences from 182 strains of 11 morphologically distinct taxa within *Skeletonema*. For details of analysis and parameter settings, see Methods. "- -" denotes NJ-bootstrap value <50%. Outgroups have been pruned away from the tree topology. Black squares, discs, triangles and starlets to the right of clades correspond to genotype groups presented in Figure 2 and mapped in Figure 4.

above 70% (Fig. 1). Multiple strains for individual species showed sequence variation and the amount of variance differed among species. *Skeletonema costatum* s. s*., S. dohrnii,* and *S. grethae* were paraphyletic; all others were monophyletic. The three markedly distinct LSU rDNA genotype groups of *S. menzelii* still formed a

single clade as did the two of *S. tropicum. Skeletonema pseudocostatum* and *S.* cf. *pseudocostatum* formed an unsupported clade (<70% bs). The large majority of strains in the *S. marinoi* clade shared the same LSU rDNA genotype or a single step away. Yet, five out of seven strains collected by Godhe et al. (2006) at Kalmar (KAL) formed a clade, so did two of the ones from Koljofjord (KF), and so did all from Georgia Strait (CAN).

Genotype networks were inferred for species with more than four sequences (Fig. 2(A–E)). *Skeletonema marinoi* had high variation and some sequences formed a reticulated pattern (Fig. 2(A)). Strains studied in our laboratory in Naples shared the dominant genotype or differed by one step from that genotype. Conversely, 13 of the 25 sequences published in Godhe et al. (2006) were several steps away from the dominant genotype. Most of the Kalmar (KAL) and Georgia Strait (CAN) strains were recovered in a reticulum. The sequences of *S. dohrnii* also formed a reticulum that was close to the dominant genotype of *S. marinoi*; the SCDT01 genotype was only two steps away.

Skeletonema costatum s. s. genotypes showed reticulation and sequences were affected by ambiguities (S, M, Y, R, K, and W) (Fig. 2(B)). *Skeletonema costatum* s. s. genotypes were at least eight steps away from *S. subsalsum*. The pattern for *S. menzelii* was completely resolved (Fig. 2(C)). Genotypes within two of the three groups were identical and differed from each other by six substitutions. The third group had two genotypes that differed by only two steps. The pattern was also completely resolved for *S. grethae* and its nearest sister, *S. japonicum* (Fig. 2(D)). The dominant genotypes of both species were only five steps from each other. *Skeletonema grethae* had two additional genotypes, one and two steps away from the dominant one. The *S. japonicum* sequence of the Chile strain was one step from the dominant genotype; this genotype was not shown in Figure 1 because the change occurred in positions excluded at the 5′-end of the alignment. The pattern for *S. tropicum* was completely resolved; the two groups were separated by at least four substitutions (Fig. 2(E)).

Biogeography

Our strain collection sites and those of Godhe et al. (2006) and Chen et al. (2007) are shown in Figures 3 and 4 (also see Table 1), which also
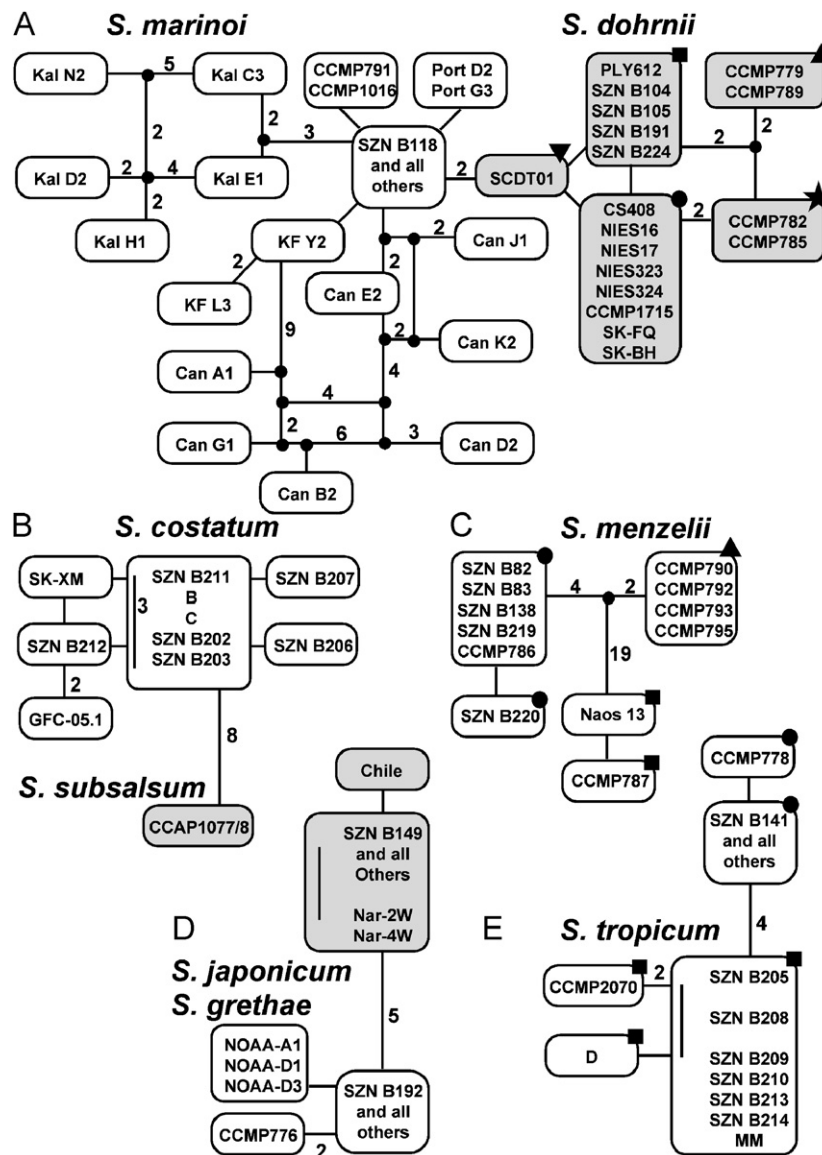
Figure 2. LSU rDNA genotype networks of morphologically delineated *Skeletonema* species. Boxes include strains sharing identical genotypes, or in case spaces intersperse the strain identifiers, two or more genotypes and sequences showing ambiguity among the included genotypes. In the latter case, the bar to the left indicates the number of steps between the most distinct sequences connected via ambiguities. Lines between boxes indicate minimum number of steps between genotypes; numbers denote steps; no number signifies a single step. Black squares, discs, triangles and starlets to the upper right of boxes correspond to genotype groups mapped in Figures 1 and 4. (**A**) S. *dohrnii* and S. *marinoi*. (**B**) S. *costatum* and S. *subsalsum*. (**C**) S. *menzelii*. (**D**) S. *grethae* and S. *japonicum*. (**E**) S. *tropicum*.

include a number of records from unambiguously identifiable photographic material (grey dots; excluded from the counts and from the list in Table 1). In some cases several strains belonging to the same species were isolated from a single net sample; after deleting these replicates, the total number of strains fell from 184 to 112. Strains isolated from a single sample generally belonged to the same species. However, two species were isolated from single samples collected in the Indian River Lagoon (Florida) and the Gulf of Naples (Italy), and three species were
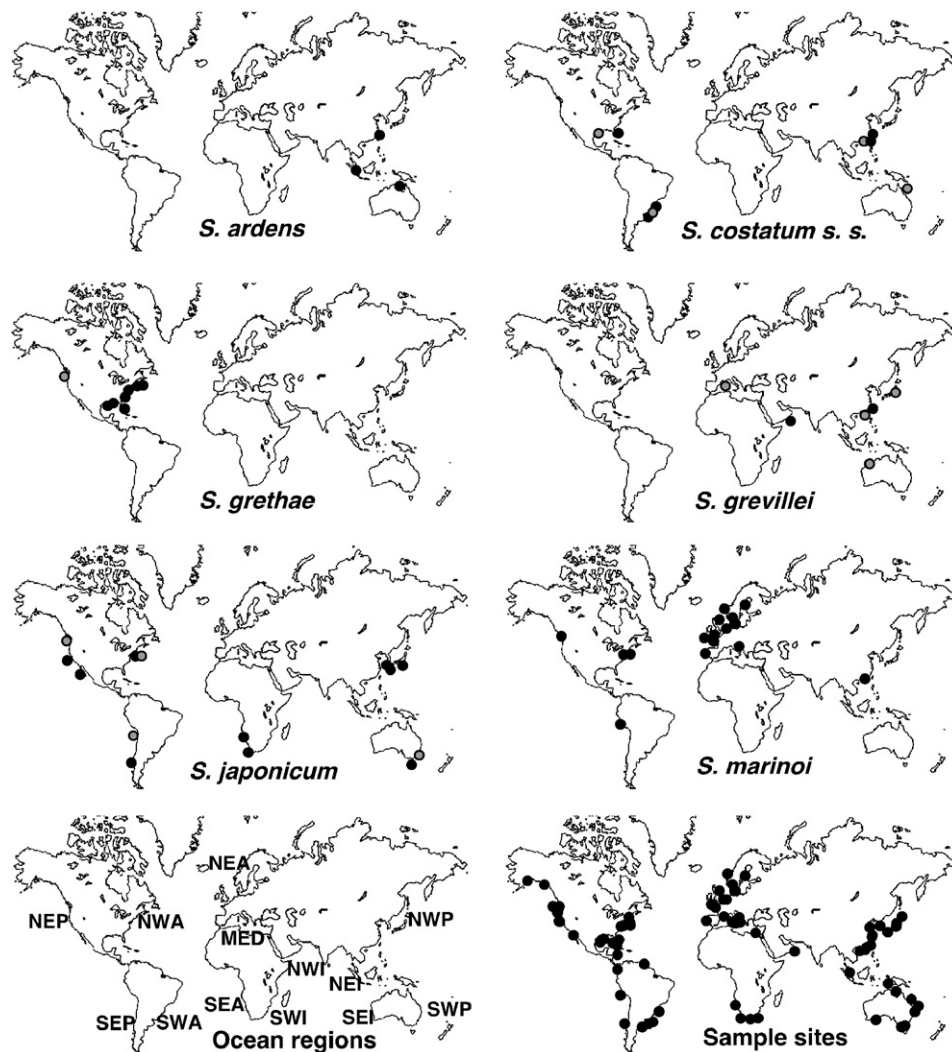
Figure 3. Distribution maps showing sample sites of six *Skeletonema* species. Black dots denote specimens identified with both ultra-structural and LSU rDNA sequence data. Grey dots denote records identified using morphology only, or morphology and SSU rDNA sequence information. Grey dots of *S. costatum* s. s.: Hong Kong, type material of Greville (Zingone et al. 2005); northern Queensland, EM illustration in CSIRO folder; Corpus Christi lagoon, Texas, SEM illustration in http://www.tamuk.edu/chemistry/research/ Analytical%20Lab/ana_lab.htm (accessed 2 Nov. 2007); Cananeia, southern Brazil (Jan-Feb 1967), material in permanent slide, collection of Grethe Hasle. Grey dot of *S. grethae*: Strait of Georgia, British Columbia, SSU-data and morphology of CCAP1077/4 (Alverson and Kolnick 2005). Grey dots of *S. grevillei*: Hong Kong, type material (Zingone et al. 2005); off Port Hedland, Western Australia (picture provided by Hallegraeff, personal communication); Sagami Bay, Japan 35°0.11′N 139°28.32′E, 23 August 1999 (Tanimoto et al. 2003); Valencia, Spain (Assadi, personal communication). Grey dots of *S. japonicum*: Peruvian upwelling zone (Hasle 1973); off the Washington coast (Jordan and Takahashi, personal communication); Narragansett Bay, SSU-data and morphology of strain NB02-45 collected 23/01/2003 (Alverson and Kolnick 2005); off Sydney (Hallegraeff, personal communication). Ocean regions: NWP, North western Pacific Ocean; SWP, South western Pacific Ocean; NEP. North eastern Pacific Ocean; SEP, South eastern Pacific Ocean: NWA, North western Atlantic Ocean; SWA, South western Atlantic Ocean; NEA, North eastern Atlantic Ocean; SEA, South eastern Atlantic Ocean; MED, Mediterranean Sea; NWI, North western Indian Ocean; SWI, South western Indian Ocean; NEI, North eastern Indian Ocean; SEI, South eastern Indian Ocean.
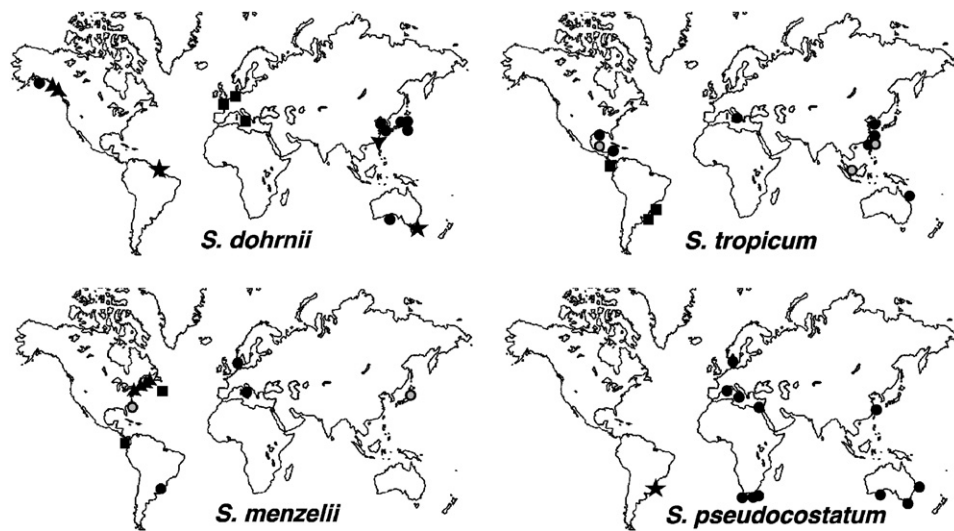
Figure 4. Distribution maps showing sample sites of morphologically defined species exhibiting different genotypes: *S. dohrnii, S. menzelii, S. pseudocostatum* (including *S.* cf. *pseudocostatum*) and *S. tropicum*. Distinct genotypes have been marked with symbols also used in Figures 1 and 2. Symbols filled in black denote specimens identified with both ultra-structural and LSU rDNA sequence data. Grey dots denote records identified using morphological data only. Grey dots of *S. menzelii*: Japan (Takano 1981); Indian River Lagoon, Florida (Badylak and Phlips 2004). Grey dots of *S. tropicum*: southern Gulf of Mexico (Castillo et al. 1995); Singapore (Hallegraeff, personal communication); Hong Kong, LM-illustration showing multiple chloroplasts and 1-to-1 intercalary fultoportular processes.

isolated from single samples at the Montevideo and Marsdiep sites. Up to four species were obtained from sites revisited in different seasons.

Skeletonema dohrnii and *S. marinoi* were found at the highest number of sites (16 and 17 excluding unknown collection sites). These two species were recorded in the northern and southern temperate zones, which were also the most densely covered regions in our sample scheme. Towards the lower latitudes the two species were found in winter or at upwelling sites, whereas they were encountered in spring or early summer at higher latitudes (Table 1). For instance, *S. dohrnii* and *S. marinoi* were found in the Mediterranean Sea in February—March but in coastal waters of northern Europe during April—July. Remarkably, the two species were found in the same area only in one case, i.e., a tidal channel between the open North Sea and the brackish Wadden Sea (Texel, The Netherlands).

Skeletonema grethae, S. pseudocostatum, S. japonicum, S. tropicum and *S. menzelii* were present at fewer sites (7, 11, 10, 11, and 9, respectively). *Skeletonema grethae,* a warm water species, was collected in Florida and the Gulf of Mexico during January—February and in Narra-

gansett Bay, USA, during May—August. The collection date of a *S. grethae* strain CCAP1077/4 from British Columbia (Alverson and Kolnick 2005) is unknown. *Skeletonema pseudocostatum* was collected in Australian, South African, and Chinese waters as well as the Mediterranean and Baltic Seas. The genetically distinct *S.* cf. *pseudocostatum* was obtained from southern Brazilian waters. *Skeletonema japonicum* was collected in cold temperate waters, either in winter (e.g., Narragansett Bay, USA in December; New South Wales, Australia in July) or in areas under influence of cold currents or upwelling (western South Africa, Namibia, Chile, Baja California). *Skeletonema tropicum* was found in tropical locations as well as in the Mediterranean Sea, the East China Sea and in the Lagoa dos Patos (southern Brazil) during summer and autumn. The only *S. tropicum* strain collected in the temperate zone in winter (SCND03, Chinese waters) was cultured from a resting stage collected from a sediment sample.

Skeletonema ardens, S. costatum s. s., and *S. grevillei* had wide geographical distributions but they were rarely collected (3, 5, and 2, respectively). In the Mediterranean Sea, *Skeletonema grevillei* was observed off Valencia (Spain)

(C. Assadi, unpublished data), but not at our plankton monitoring station in the Gulf of Naples.

The distinct genotype groups within morphologically identical species had allopatric distributions (Fig. 4). One *S. tropicum* genotype was collected from the coasts of the Eastern USA, China, and North-eastern Australia as well as the Mediterranean Sea; the second group was collected along the coasts of Uruguay, Brazil, and the Pacific side of Panama. For *S. menzelii*, one genotype was found along the East coast of North America, a second from European and Uruguayan waters, and a third from the Gulf of Panama and the Sargasso Sea. The strain from the Sargasso Sea corresponds to the type material of the species (Guillard et al. 1974). And finally, for *S. dohrnii*, one genotype came from western Europe and the Mediterranean Sea, a second from the coasts of Japan, China, southern Australia, the Alaskan panhandle, and California, a third from Alaskan and British Columbian waters, and the fourth from Bass Strait, Australia and the tropical western Atlantic (off Suriname).

Discussion

Species Delineation in *Skeletonema*

The species delineated in previous studies (Sarno et al. 2005, 2007) had distinct morphological features supported by consistent differences in both SSU and LSU rDNA. The present study also shows that *Skeletonema* species generally retain their morphological and genetic distinctness even when the sample collection size is increased drastically and when specimens are gathered from distant places. Apparently, the genus is not composed of a single genetically diverse metapopulation sensu Finlay and Fenchel (2004).

Nonetheless, there are exceptions. Firstly, *S. dohrnii* and *S. marinoi* are not always distinguished using morphology alone, as suggested by Sarno et al. (2005), because of overlap in girdle band ultrastructure. Chen et al. (2007) report strains that belong genetically within *S. dohrnii* but have subtle morphological differences from *S. dohrnii*. At times, we noted similar morphological deviations as observed by the latter authors in miniaturized strains. Furthermore, although *S. dohrnii* and *S. marinoi* are reciprocally monophyletic in the unrooted network in Figure 2, their most proximal genotypes are only two steps apart, and the *S. dohrnii* group does not possess any synapomorphies in Figure 1. Based on previous criteria (Sarno et al. 2005, 2007) the two taxa would not be separated. Three hypotheses can be formulated concerning *S. dohrnii* and *S. marinoi*: (1) they could constitute a single, genetically diverse species (sensu Fenchel 2005); (2) they could be two different species, with *S. marinoi* being monophyletic inside a paraphyletic *S. dohrnii*, or (3) they represent several species, including a monophyletic *S. marinoi* and several cryptic species in *S. dohrnii*. In the *Bostrychia radicans* (Montagne) Montagne—*B. moritziana* (Sonder ex Kützing) J. Agardh species-complex (Rhodophyta), differences in geographical ranges reflect genetically distinct and reproductively isolated species (Zuccarello and West 2003; Zuccarello et al. 2006). An analogous separation among *S. dohrnii-marinoi* clades is suggested by distribution patterns (Sarno et al. 2005, confirmed here). However, *S. dohrnii* was not well represented among our isolates and we reserve a final taxonomic interpretation until there is intensive sampling at a regional scale and there is information from more sensitive molecular markers.

A second set of problematic cases is the genotype groups within *S. tropicum* and *S. menzelii*, and the genetically distinct groups of *S. pseudocostatum* and *S.* cf. *pseudocostatum*. The clades probably constitute cryptic species, but again we abstain from proposing taxonomic changes until more data are available. *Skeletonema tropicum* and *S. menzelii* are still monophyletic taxa whereas monophyly is not even clear for the group with *S. pseudocostatum* and *S.* cf. *pseudocostatum*. A third problem is that the sequences of *S. grethae* and *S. costatum* s. s. strains form grades. Nonetheless, we believe that both are valid species because their intraspecific LSU rDNA sequence variation is small. In addition, we are in the process of sequencing a series of plastidial DNA sequences and preliminary phylogenies inferred from these markers reveal monophyly for *S. grethae* and *S. costatum* s. s., though *S. dohrnii* remains paraphyletic (unpubl. observ.).

These taxonomic problems indicate that no single set of criteria currently defines species in *Skeletonema*. This may be partially due to our limited analytical tools and restricted sample sizes. For instance, the LSU rDNA region is a multi-copy marker and intra-individual polymorphism could potentially hamper species delineation. Yet, Alverson and Kolnick (2005) showed that directly sequenced PCR products resolved in the base of species clades. They did not find any ancestral sequences that broke down

the distinction between species clades composed of sequences obtained by direct sequencing.

Sequence heterogeneity results not only from random single base substitutions but probably also from more extensive changes. Clusters of base changes cause the long branches of *S. grevillei, S. ardens,* one of the lineages in *S. menzelii* (Sarno et al. 2007) and probably the clade with *S. tropicum, S. pseudocostatum* and *S.* cf. *pseudocostatum* (Fig. 1). Each cluster may result from a single event of multiple concurrent changes that was accidentally swept to dominance by means of concerted evolution and bottlenecks in the populations (Sarno et al. 2007).

The presence or absence of intra-specific variation in some morphologically delineated species can be explained in different ways. The genetic differences between Western Europe and the North Pacific, as observed in *S. dohrnii*, suggest that long distance gene flow is probably small enough to permit the emergence of genetic differences among regions by means of mutations and random drift. Dispersal might not be as massive and far-reaching as has been suggested for microbial organisms (e.g., Finlay 2002). Clades within *S. menzelii* and *S. tropicum* may also have arisen by geographical isolation. Conversely, intra-specific variation is apparently lacking in *S. japonicum*, which suggests unimpeded long-range dispersal or recent range expansion.

Genetic structure seems to differ even among different geographical sites within a taxon. *Skeletonema marinoi* from Kalmar and Georgia Strait have high LSU rDNA variation (Godhe et al. 2006), but there is little or no genetic variation among strains from elsewhere (including the Portuguese strains of Godhe et al. 2006, the Adriatic ones of Sarno et al. 2005, and a set of 21 strains collected in the latter region during the spring bloom of 2007 by Casotti et al. unpublished data). These differences in intra-specific genetic variation among sites might result from different reproduction modes. Strictly clonal reproduction can be correlated with considerable intraspecific genetic variation in many protists (Koch and Ekelund 2005; Nanney et al. 1998). Macroalgal populations, at the fringes of their distribution ranges, often consist largely or entirely of clones (Scrosati and Servière-Zaragoza 2000; Scrosati 2004). Most diatoms must have sexual reproduction to escape frustule miniaturization (Round et al. 1990). Although sex has been reported for *Skeletonema* (Migita 1967), members of this genus can restore their maximum size through vegetative cell enlargement (Gallagher 1983), thus allowing their populations to persist as genetically distinct clonal cell lines. Therefore, the genotypically distinct *S. marinoi* strains from Kalmar and Georgia Strait may represent long-lived, distinct clonal or selfing cell lines whereas the genetically homogeneous strains may exhibit regular sexual reproduction. Species with little or no LSU-genotype variation (e.g., *S. pseudocostatum* and *S. japonicum*) may constitute widespread but sexually interbreeding populations, or alternatively, we simply missed the clonal populations for these species.

Biogeographical Patterns in *Skeletonema*

Despite some limitations (e.g., only 184 strains), it is clear that most *Skeletonema* species are widely distributed throughout either temperate or tropical coastal regions. For example, *S. japonicum* occurs in the cool temperate coastal regions, and *S. tropicum* has a warm water distribution. Based on physiological experiments and on in situ observations, Hulburt and Guillard (1968) concluded that *S. tropicum* could not exist where seawater winter temperatures drop below 15 °C. Our records extend this range slightly because *S. tropicum* occurred in the Mediterranean Sea and coastal waters of Uruguay where winter seawater surface temperatures are as low as 12—14 °C. *Skeletonema grethae* is apparently the only exception to the widespread distribution of species because it was found only along the Atlantic side of the USA. Alverson and Kolnick (2005) identified strain CCAP1077/4 from the cold temperate Georgia Strait, British Columbia, as *S. grethae*. We found *S. grethae* only in warm water along the southern and eastern coasts of the USA. Either we have failed to detect this species in cold waters, or this species occurs in seasonally warm estuarine habitats all over the globe, and we have failed to detect it so far, or a culture mix-up occurred (see below). *Skeletonema pseudocostatum* seems to be lacking from the Americas, but this might be an artefact of the relatively low number of strains obtained from this species.

Despite the general distributions, some interesting patterns occur. *Skeletonema dohrnii* and *S. marinoi* were collected many times from temperate zones but they were found at the same site only once (Marsdiep, The Netherlands), which is suggestive for some form of ecological differentiation. To our surprise, we did not encounter a single strain of *S. subsalsum* in our field samples. We did not sample extensively in brackish waters,

the typical habitat of the species (Hasle and Evensen 1975), but nevertheless, many previous observations suggest that the species is not rare. *Skeletonema subsalsum* and *S. costatum* s. s. are highly similar in LM and perhaps *S. subsalsum* was misidentified in some earlier reports. *Skeletonema costatum* s. s. and *S. grevillei* were found at very distant sites and they were infrequently collected, which suggests that they have wide distribution ranges. Similarly, the tropical gap in the *S. japonicum* distribution and the disjunctive Atlantic—Indo-Pacific distribution of *S. tropicum* suggest that these strains also disperse over long distances, apparently through unfavourable regions. Vicariance and dispersal biogeography as well as recent man-made dispersal routes and ballast water transport may account for the geographical distributions (e.g., Hallegraeff 1998; McMinn et al. 1997; Nehring 1998; Zingone et al. 2003). Clades of *S. tropicum*, *S. menzelii,* and *S. dohrnii* had mutually exclusive geographical ranges that may indicate slow dispersal, allowing for the emergence and persistence of genetic differences or even permitting allopatric speciation to occur. Such hypotheses are testable using population genetic approaches, but unfortunately, our data are too crude for this problem.

Seasonality and regional differences are known for phytoplankters, e.g., Rynearson and Armbrust (2005) and Rynearson et al. (2006) found genetically different populations of *Ditylum brightwellii* (T. West) Grunow in Van Heurck in adjacent water masses separated only by hydrographical boundaries or seasons of the year. In our study, *Skeletonema dohrnii* occurred in the Gulf of Naples only during the winter and spring, and it was absent from the Adriatic samples; *S. marinoi* was found in the northern Adriatic but it was never found in the Gulf of Naples.

Identification of *Skeletonema* Strains in Culture Collections

Our investigations resulted in a re-identification of *Skeletonema* strains kept in culture collections around the world. These re-identifications will allow others to reinterpret physiological and other experimental studies that were attributed to a single, cosmopolitan species. Furthermore, because culture collections may occasionally mislabel cultures, additional confusion has occurred. For example, *S. dohrnii* strain CCMP789 was collected from the tropical Atlantic Suriname coast, whereas all other strains of this species occurred in cool water. The strain was originally identified as *S. tropicum*, a species that is easily recognized by its numerous chloroplasts, and furthermore, CCMP789 has an identical LSU rDNA sequence to that for CCMP779, which was collected from Bass Strait, Tasmania. The identity of strain CS-76 is also problematic. Medlin et al. (1991) prepared type material of *S. pseudocostatum* using strain CS-76, and they found this strain to be genetically identical to CCAP1077/7. We sequenced the LSU rDNA and examined the frustule ultrastructure twice using material from two independent shipments, and we found that strain CS-76 was *S. japonicum*. A strain mix-up must have occurred, between the Medlin et al. (1991) and our studies (Sarno et al. 2005, this study), perhaps involving the discontinued strain CS-75.

Methods

Strain information and GenBank accession numbers for partial LSU rDNA sequences are listed in Table 1. Sixty strains were obtained from culture collections (AC, CCAP, CCMP, CS (CSIRO), CTCC, MBA, NIES, SCXM); unless stated otherwise the remainder was isolated into culture from single chains found in plankton net-samples. Isolation, growth and morphological examination are described by Sarno et al. (2005). Strain identification details are described in Sarno et al. (2005, 2007) and Zingone et al. (2005). DNA extraction, PCR amplification and LSU rDNA alignment are described in Sarno et al. (2005). Sequence products were obtained with the BigDye Terminator Cycle Sequencing technology (Applied Biosystems, Foster City, CA) and purified in automation using a robotic station "Biomek FX" (Beckman Coulter, Fullerton CA). Products were analysed on an Automated Capillary Electrophoresis Sequencer "3730 DNA Analyzer" (Applied Biosystems). Any site showing an ambiguity in the forward and reverse reading frame was recorded as such if surrounding sites read without any difficulties, even if one of the bases dominated the other. The LSU rDNA alignment is available under study — and matrix accession numbers on S1901 and M3491, respectively at TreeBASE http://treebase. org/treebase (pin 30601).

Phylogenetic analyses were carried out in PAUP* version 4.0b10 (Swofford 2002). The alignment of the 182 *Skeletonema* LSU rDNA sequences and six outgroup sequences (*Cyclotella meneghiniana* Kützing AY496205, *Cyclotella* sp. G18W42 AY496198, *Thalassiosira rotula* Meunier CCAP1806 AJ633505, *T. rotula* CCMP1647 EF423391, *T. rotula* CCMP1018 EF423392, and *Thalassiosira* sp. SZN B101 AJ633506) included 829 positions, from which the first ten and the last nine were omitted for phylogenetic analysis because the 5′-end and the 3′-end were incomplete in many of the sequences. Neighbour joining trees were inferred with pairwise ML-distances constrained with base substitution parameter settings as used in Sarno et al. (2007) for the LSU rDNA (base frequencies: $A = 0.2667$, $C = 0.1955$, $G = 0.2530$, $T = 0.2848$; substitution rates $A \Leftrightarrow C = 1.1187$, $A \Leftrightarrow G = 2.8531$, $A \Leftrightarrow T = 1.2761$, $C \Leftrightarrow G = 1.2705$, $C \Leftrightarrow T = 4.7871$, relative to $G \Leftrightarrow T = 1.0000$; proportion of invariable

sites = 0.5377; rates gamma distributed with shape para-meter alpha = 0.8081). Bootstrap values were calculated with 1000 replicates under the same settings as in the NJ analysis. Note that the purposes of the phylogenetic approach are to address the phylogenetic status of morphologically deli-neated groups of strains and assess if these groups form clades or grades.

Genotype networks were obtained for groups of LSU rDNA sequences belonging to the same morphologically circum-scribed species using TCS 1.21 (Clement et al. 2000). Since this programme cannot handle ambiguities, an ambiguity, e.g., an R, at a given position was treated as a G if other sequences showed only an A at that position. In case sequence had, e.g., an R at a given position and the other sequences showed either a G or an A at that position, a box was drawn around all the strains having an A, a G or an R at that position and the difference was indicated with a vertical bar inside the box.

Acknowledgements

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