New Phytologist

Genetic diversity of *Ectocarpus* (Ectocarpales, Phaeophyceae) in Peru and northern Chile, the area of origin of the genome-sequenced strain

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Received: *21 January 2010*
Accepted: *6 April 2010*

New Phytologist (2010) **188**: 30–41
doi: 10.1111/j.1469-8137.2010.03303.x

Key words: Chile, *Ectocarpus*, genetic diversity, *Kuckuckia*, Peru.

Summary

- The origin of the *Ectocarpus* strain used for genome sequencing (the 'genome strain') was Peru, where no *Ectocarpus* had been reported previously. To study the genetic diversity in the region and to increase the number of individuals from this area available for genetic experiments, 119 new *Ectocarpus* strains were isolated from eight localities along the 3000 km of coastline from central Peru to central Chile.
- Internal transcribed spacer 1 (ITS1) genotyping revealed nine different genotypes, five of which were endemic to the area studied and three of which were previously unknown.
- Individuals of the same genotype as the genome strain occurred from Peru to northernmost Chile, representing 61% of the samples in this area, from which five more genotypes were isolated. Further south, down to central Chile, most individuals belonged to *Ectocarpus siliculosus*, *Ectocarpus fasciculatus* and *Ectocarpus crouaniorum*. In sexual crosses, the genome strain and the new isolates of the same genotype were fully compatible.
- Sequences from four nuclear and cytoplasmic genetic markers (ITS1, ITS2, Rubisco spacer and Cytochrome-c oxidase subunit 3 (*cox*3)) separated the genome strain from the known species of *Ectocarpus*. It may in future be recognized as a separate species.

Introduction

The *Ectocarpus* strain chosen for genome sequencing (Cock *et al.*, 2010) is a male gametophyte which has the designation 'Ec32' in the *Ectocarpus* strain collection at Roscoff (France). Henceforth it will be referred to as the 'genome strain'. It was selected because it displays an alternation of two morphologically distinguishable generations (Peters *et al.*, 2008) and produces unilocular sporangia on parthenogenetic sporophytes, both of which facilitate studies of the complex brown algal life history (for more information and for the terminology used in morphological and life history descriptions, see Peters *et al.*, 2004a; Coelho *et al.*, 2007; Charrier *et al.*, 2008). The genome strain was obtained from a meiospore of a field sporophyte collected by the first author (A.F.P.) in November 1988 at San Juan de Marcona, Peru (site 3 in Fig. 1). It was identified as *Ectocarpus siliculosus* (Dillwyn) Lyngbye in an initial molecular phylogeny (lineage 1c in Stache-Crain *et al.*, 1997; strain designation 'SAm120h'), but it showed post-zygotic incompatibility (Peters *et al.*, 2004b) when crossed with a strain of *E. siliculosus sensu stricto* (genetically close to those used by Müller (1967) for the description of the life history; lineage 1a in Stache-Crain *et al.*, 1997) or with a strain of *Ectocarpus* from New Zealand (Müller, 1991; lineage 4 in Stache-Crain *et al.*, 1997): hybrids possessed a normal

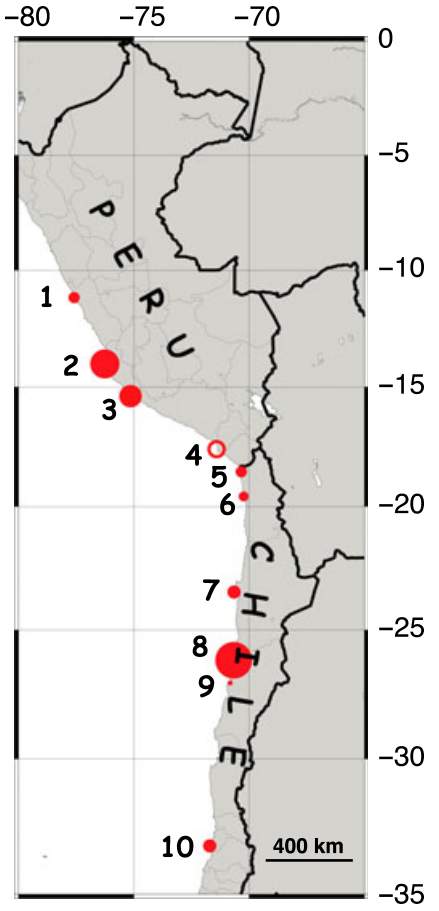

Fig. 1 Origin of isolates for the present study. See Table 1 for details of collecting sites. Diameters of closed circles are equivalent to the number of isolates from each site. No *Ectocarpus* was found at the highly exposed site 4 (open circle). The genome strain is a descendant of a field sporophyte collected in 1988 at site 3. The isolate from site 9 was available from the Culture Collection of Algae and Protozoa (CCAP), Oban, UK. The map was generated using http://www.aquarius.ifm-geomar.de/omc/.

morphology, but were unable to form meiospores. Further crosses of the genome strain with an *Ectocarpus* from northern Chile, as well as with *Ectocarpus crouaniorum* Thuret in Le Jolis from France, gave similar results (A.F. Peters, unpublished data). Although sisters of the genome strain (female gametophytes from the same field sporophyte from Peru) were available for genetic experiments, for instance to test sex-linkage of a mutation (Peters *et al.*, 2008), no female gametophyte of sufficient genetic distance was available for experiments requiring outcrossing (e.g. to produce a genetic map; Heesch *et al.*, 2010).

Apart from the genome strain there was no unambiguous published record of *Ectocarpus* from Peru. Howe (1914: p. 50) mentioned 'small specimens of *Ectocarpus*' on *Desmarestia peruviana* Montagne at Ancón (13 February 1907) and on *Lessonia nigrescens* Bory de Saint-Vincent at Chincha Island (18 June 1907); however, he regarded the material as 'too meagre to justify an attempt at determination or description'. The nearest published record of *Ectocarpus* was from Iquique, northern Chile, at *c.* 800 km distance from site 3 (Ramírez & Santelices, 1991).

The aim of this study was to isolate *Ectocarpus* strains that were fully compatible with the genome strain. Individuals were collected along the South American Pacific coast from central Peru to central Chile and characterized based on sequence comparisons with 43 strains of *Ectocarpus* and seven of *Kuckuckia* (the sister genus of *Ectocarpus*) from all continents except Antarctica (Stache-Crain *et al.*, 1997). Our experiments revealed a surprisingly high genetic diversity in *Ectocarpus* in the studied area, indicating that additional species will possibly have to be established within this genus in the future. Isolates of one of the nine genotypes found were genetically similar to the genome strain and proved to be fully interfertile with it, providing a polymorphic outcrossing line that has been used elsewhere for genetic analyses.

Materials and Methods

Field collection and isolation of cultures

Nine sites (four in Peru and five in Chile) spanning a distance of *c.* 3000 km were visited for collection, most during one visit in February–March 2006 (Fig. 1, Table 1). Sampling at site 8 was performed for studies on the response of *Ectocarpus* to copper pollution (A.D. Mann *et al.*, unpublished data). From each field thallus macroscopically resembling *Ectocarpus*, filaments were inoculated into a 2-ml Eppendorff tube containing autoclaved sea water. After transfer to the Biological Station at Roscoff, isolation and cultivation of clean unilalgal clonal cultures were undertaken as described previously (Peters *et al.*, 2010). A number of thalli from site 8 were isolated by filtering nearshore surface seawater and cultivating filaments that developed on filter paper, or by inoculating field macrothalli of *Scytosiphon* into culture and isolating all *Ectocarpus* thalli developing on them. Only thalli possessing ribbon-shaped plastids (which are diagnostic for the genera *Ectocarpus* and *Kuckuckia*) were retained for further study.

Strain designations beginning with 'CCAP' are from the Culture Collection of Algae and Protozoa (held at the Scottish Marine Institute; Oban, UK); strain designations beginning with numbers or 'Ec' are isolates housed in the *Ectocarpus* strain collection (held at Roscoff), and are maintained by the first author (A.F.P.).

Characterization of strains

Ectocarpus taxonomy is currently being updated, with major modifications being made to the existing classification system. The two-species concept, recognizing only *E. siliculosus* and *Ectocarpus fasciculatus* Harvey (Russell,

Table 1 Origin and number of isolates

Site no.	Locality	Date	Coordinates		No. isolates	Genotypes present (number of isolates)	ITS1 sequenced (*n*)	Site details[1]		Collection details	Strains[2]
1	Ancón	7 March 2006	−11.775	−77.186	4	GT4 (4)	3	Playa de Ancón Playa San Francisco	Sheltered Sheltered	Drift, on *Gracilaria*	311–314
2	Bahía Mendieta (South of Paracas)	8 March 2006	−14.063	−76.269	31	GT4 (28)	1	Playa Mendieta	Sheltered	Drift, on various macroalgae	201–202, 246–247, 266–267, 269–271, 274–285, 289–293, 315–316
			−14.061	−76.273		GT1 (3)	2	Cueva de la Zorra	Mid-exposed	Upper subtidal, on phylloid of *Macrocystis*	286–288
3	San Juan de Marcona	9 March 2006	−15.356	−75.172	16	GT4 (2)	1	Playa Hermosa	Sheltered	Drift, on *Desmarestia peruviana*	301, 303
						GT2 (12)	7	Playa Hermosa	Sheltered	Drift, on various macroalgae	294–300, 302, 304–307
			−15.361	−75.189		fas (2)	1	Reserva Punta San Juan	Mid-exposed	Upper subtidal, on phylloid of *Macrocystis*	308–309
4	Ilo	10 March 2006	−17.625	−71.344	0	None	0	Playa Boca del Río	Highly exposed	–	–
			−17.700	−71.380		None	0	Punta Coles		–	–
5	Arica	1 March 2006	−18.489	−70.328	5	GT4 (2)	2	South end of town	Mid-exposed	Subtidal 3–5 m, on *Lessonia trabeculata* and red macroalga	161, 721
						fas (1)	1	South end of town	Mid-exposed	Subtidal 3–5 m, on *Lessonia trabeculata*	310
			−18.481	−70.330		fas (1)	1	Yacht harbour	Sheltered	Upper subtidal, on *Lessonia trabeculata*	165
						Kuck (1)	1	Yacht harbour	Sheltered	Upper subtidal, on rope	160
6	Pisagua	2 March 2006	−19.567	−70.207	3	GT1 (1)	0	Cementerio	Exposed	Drift, on *Lessonia nigrescens*	159
			−19.597	−70.216		GT1 (1)	1	Shore near wharf	Exposed	Intertidal, on *Lessonia nigrescens*	157
						GT3 (1)	1	Shore near wharf	Exposed		156
7	Antofagasta	28 February 2006	−23.476	−70.608	6	sil (5)	1	Bolsico (N of town); beach near *Gracilaria* bed	Sheltered	Drift	150–151, 153–155
			−23.759	−70.465		sil (1)	1	Coloso (S of town): artificial rocky shore	Sheltered	Intertidal	147

Table 1 (Continued)

Site no.	Locality	Date	Coordinates		No. isolates	Genotypes present (number of isolates)	ITS1 sequenced (*n*)	Site details[1]		Collection details	Strains[2]
8	Chañaral	February 2005	−26.258	−70.649	47	GT1 (1)	1	Soldado (N of copper discharge point)[3]	Sheltered	Subtidal, from spore suspension	521
		January 2004	−26.275	−70.661		sil (16)	2	La Lancha and Palito (close to copper discharge point)[3]	Exposed	Intertidal or developed in culture on *Scytosiphon*	466, 509–512, 524–525, 606, 614–615, 621–623, 630–632
		February 2005	nd	nd		fas (12)	2	Different sites, all without copper pollution	Exposed	Subtidal, from spore suspension, or intertidal	523, 526, 528, 607, 611, 620, 624–629
		January 2004				cro (16)	3	La Lancha and Palito (close to copper discharge point)[3]	Exposed	Intertidal or developed in culture on *Scytosiphon*	514–520, 527, 608, 610, 612–613, 616–619
		January 2004				sil + cro (2)	0	La Lancha and Palito (close to copper discharge point)[3]	Exposed	Intertidal or developed in culture on *Scytosiphon*	513, 609
9	Caldera[4]	2 October 1990	−27.102	−70.860	1	GT2 (1)	1	*Gracilaria* farm	Sheltered	On *Gracilaria*	CCAP1310/40
10	Quintay	5 October 2006	−33.193	−71.702	7	GT2 (1)	1	Marine Station of Universidad Andrés Bello	Sheltered	Large tide pool, saxicolous	639
						fas (6)	2				633–638
	Total				120		36				

ITS, internal transcribed spacer; Kuck, *Kuckuckia*; sil, *Ectocarpus siliculosus*; fas, *Ectocarpus fasciculatus*; cro, *Ectocarpus crouaniorum*; nd, no data.
[1]Exposure was estimated during collection.
[2]Designation in *Ectocarpus* strain collection at Roscoff or Culture Collection of Algae and Protozoa (CCAP), Oban, UK.
[3]For a description of the site see Andrade *et al.* (2006).
[4]Strain collected by Mariela Gonzales and isolated by Dieter G. Müller.

1966, 1967; Stache-Crain *et al.*, 1997), has recently been rejected following the reinstatement of a third species, *Ectocarpus crouaniorum*; additional lineages so far included in *E. siliculosus* or *E. fasciculatus* may equally deserve species rank (Peters *et al.*, 2010). In the present paper the taxa *E. siliculosus* and *E. fasciculatus* are referred to in a narrow sense; that is, corresponding to lineages 1a and 5b, respectively, in Stache-Crain *et al.* (1997).

For the identification of isolates from Peru and northern Chile, we used DNA sequences. DNA was extracted from living cultures as described in Peters *et al.* (2004b). Nuclear ribosomal internal transcribed spacer (ITS) data for 50 *Ectocarpus* and *Kuckuckia* strains were available as reference sequences (Stache-Crain *et al.*, 1997). The first part of ITS1, which is 134–701 bp long and flanked by the motifs GATCATTACCGA and TTATYGGTYGGG, is particularly useful to distinguish genotypes because it is highly variable and indel-rich. Initial characterization of all strains was based on ITS1 length (as in Peters *et al.*, 2010), followed by sequencing of ITS1 in 30% of the isolates or by a diagnostic polymerase chain reaction (PCR) using a primer specific for the ITS1 sequence of the genome strain (for details and oligonucleotide primers employed, see Supporting Information Notes S1 and Table S1, respectively). Blast searches (Altschul *et al.*, 1997) were carried out utilizing the first parts of newly generated ITS1 sequences as queries. This allowed classification of the sequences into different ITS1 genotypes (GTs): sequences presenting ≥ 95% identity were considered to belong to the same GT. The different GTs were numbered based on their ITS1 length, the lowest number corresponding to the shortest length. For at least one member of each GT discovered, three additional markers (ITS2, 3′-Ribulose-bisphosphate carboxylase (*rbc*L) + Rubisco spacer and Cytochrome-c oxidase subunit 3 (*cox*3) were sequenced to provide data for phylogenetic analyses. Sequences were deposited in the European Molecular Biology Laboratory (EMBL)/GenBank/DNA Data Bank of Japan (DDBJ) database (Table 2).

Phylogenetic analyses

Sequences were aligned manually using Se-Al v. 2.0a11 (Rambaut, 2002). Sequences of the four markers were concatenated, excluding more variable parts of ITS, which could not be aligned with confidence. Sequences of reference strains (nine from Europe, and one each from Pacific South America and New Zealand; Table 2) were then added, and phylogenetic analyses undertaken. Phylogenetic trees were constructed using the various programs in phylip version 3.69 (Felsenstein, 1995), and the robustness of the alignments was tested with the bootstrapping option (SeqBoot). Genetic distances, applicable for distance matrix phylogenetic inference, were calculated using the Dnadist program in the phylip package. Phylogenetic inferences based on the distance matrix (Neighbor), maximum likelihood (Dnaml) and parsimony (Dnapars) algorithms were applied to the alignments. In all cases, the best tree or majority rule consensus tree was selected using the consensus program (Consense). The trees were visualized and drawn using the treeview software version 2.1 (Page, 1996).

Results

Thalli of *Ectocarpus* occurred at eight out of the nine sites surveyed, usually as epiphytes on other macroalgae such as *Macrocystis*, *Lessonia*, *Desmarestia* and *Gracilaria*. Occasionally they were saxicolous. The *Ectocarpus* individuals were present from sheltered to exposed conditions but were not found at a highly exposed site (Table 1). The maximum thallus size was 14 cm but several samples were only minute felts or dark spots; other thalli appeared in culture after inoculation with their substratum or they developed on paper that was used to filter sea water (Figs 2a–c; Table 3). A total of 120 strains, including strain CCAP1310/40 isolated previously at site 9, were available for identification of genotypes.

Five different ITS1 length types were clearly distinguishable on agarose gels, and one putative hybrid (Table 3, Fig. 3). Sequencing of 36 individuals (Table 1) revealed that two of the length types were heterogeneous and contained more than one ITS1 GT. In total we obtained nine different GTs representing seven *Ectocarpus* genotypes, one *Kuckuckia* genotype and one hybrid between two of the *Ectocarpus* genotypes (Table 3).

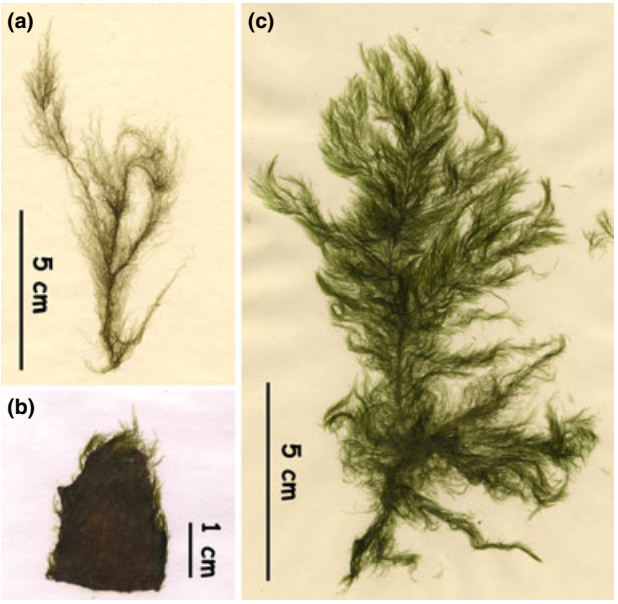

Fig. 2 Herbarium specimens of *Ectocarpus* from Peru, collected in 2006 from drift material. (a) Genotype 4 (GT4), site 1. (b) Several thalli of GT4 epiphytic on *Desmarestia firma* Skottsberg, site 2. (c) GT2, site 3.

Table 2 DNA sequences utilized in the present study

				ITS1 + 2	ITS1[1]	ITS2[1]	Rubisco spacer[2] (515 bp)	*cox*3 (665 bp)
	Species/genotype	Strain	Origin	Acc	bp	bp	Acc	Acc
1	GT1	Ec157	Site 6	**FN564453**	306	247	**FN564475**	**FN564526**
2	GT1	Ec286	Site 2	**FN564454**	302	247	**FN564476**	**FN564527**
3	GT2	Ec298	Site 3	**FN564456**	344	293	**FN564477**	**FN564528**
4	GT2	CCAP1310/40	Site 9	**FN564455**	352	292	U38736	**FN564529**
5	GT3	Ec156	Site 6	**FN564457**	347	264	**FN564478**	**FN564530**
6	GT4	Genome strain Ec32 = CCAP1310/4	Site 3	AJ550048	362	252	AJ550050	From genome project
7	GT4	Ec721	Site 5	**FN564446**[3]	362	–	–	–
8	GT5 = *E. fasciculatus*	Ec310	Site 3	**FN564458**	433	292	**FN564479**	**FN564531**
9	GT6 = *E. siliculosus sensu stricto*	Ec147	Site 7	**FN564466**[3]	716	–	–	–
10	GT7 = *Kuckuckia* sp.	Ec160	Site 5	**FN564460**	714	290	**FN564480**	**FN564532**
11	GT8 = *E. crouaniorum*	Ec608	Site 8	**FN564459**	855	253	**FN564481**	**FN564533**
12	*E. fasciculatus*	CCAP1310/12	Plouescat, France	U38824	429	286	U38711	FN564521
13	*E. fasciculatus*	fas BR1 = Ec395	Roscoff, France	FN564441	428	282	FN564468	FN564513
14	*E. fasciculatus*	fas BR2 = Ec541	Plougonvelin, France	FN564449	439	282	FN564471	FN564518
15	*E. siliculosus sensu stricto*	sil BR1 = Ec393	Roscoff	FN564440	714	247	FN564467	FN564512
16	*E. siliculosus sensu stricto*	sil BR2 = Ec540	Plougonvelin	FN564448	712	247	FN564470	FN564517
17	*Ectocarpus* sp.	CCAP1310/47[4]	Kaikoura, New Zealand	U38766	749	260	U38722	FN564523
18	*E. crouaniorum*	cro BR = Ec471	Roscoff	FN564442	865	256	FN564469	FN564514
19	*E. crouaniorum*	1310/144	Isle of Man, United Kingdom	U38771	863	256	U38726	FN564522
20	*Ectocarpus* sp.	Ec319[5]	Cherbourg, France	FN564452	644	275	FN564474	FN564519
21	*Kuckuckia spinosa*	CCAP1320/3	Villefranche, France	U38829	827	266	U38705	FN564524
22	*Kuckuckia spinosa*	CCAP1320/4	Isla Robinson Crusoe, Chile	U38825	689	267	U38709	FN564525

Newly generated sequences are shown in bold.
Acc, GenBank/European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Database/DNA Data Bank of Japan (DDBJ) accession; bp, base pairs (length); –, not applicable; ITS, internal transcribed spacer; *cox*3, cytochrome-c oxidase subunit 3.
[1]Limits: ITS1: start atcattaCCGA, end GTTGTAaaacttt; ITS2: start gtctgttGACACC, end TTTCGTTcggacct (Stache-Crain *et al.*, 1997).
[2]Including flanking ribulose-bisphosphate carboxylase large subunit (*rbc*L) and ribulose-bisphosphate carboxylase small subunit (*rbc*S) gene sequences.
[3]Only ITS1; in addition to the sequence of strain Ec147, the slightly different ITS1 sequence of a particularly copper-resistant strain from site 8 (CCAP1310/333; Ritter *et al.*, 2010) was deposited (FN564444).
[4]Strain used in Müller (1991) and Peters *et al.* (2004b); lineage 4 in Stache-Crain *et al.* (1997).
[5]Strain representing lineage 5a of Stache-Crain *et al.* (1997).

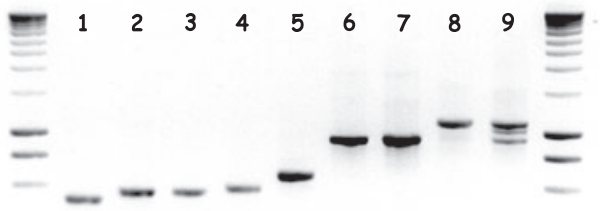

Fig. 3 Internal transcribed spacer 1 (ITS1) lengths in *Ectocarpus* and *Kuckuckia* from Peru and Chile. A 2% agarose gel was run at low voltage, and stained with ethidium bromide. Flanking lanes contain length standards (600, 800, 1000 and 1500 bp from the bottom). Lanes 1–9: lane 1, genotype (GT) 1; lane 2, GT2; lane 3, GT3; lane 4, GT4 (= genome-sequenced strain); lane 5, *E. fasciculatus*; lane 6, *E. siliculosus*; lane 7, *Kuckuckia* sp.; lane 8, *E. crouaniorum*; lane 9, putative hybrid between *E. siliculosus* and *E. crouaniorum*; the middle band is a PCR artefact. Note that lanes 2–4 and 6–7 have lengths that are hardly (if at all) distinguishable in an ordinary 1% agarose gel. See Table 3 for precise ITS1 lengths.

Individuals with the same GT as the genome strain (GT4) occurred from site 1, where they were the only *Ectocarpus* collected, to site 5 at the northern border of Chile (Tables 1,3). The ITS1 regions of the seven sequenced strains of this GT were identical to that of the genome strain (n = 2; sites 2 and 3), differed by a single nucleotide substitution (n = 4; three from site 1 and one from site 5; see Table 2 for the sequence accession of the latter strain), or had an ambiguity at the same position (n = 1; site 5).

We collected eight additional genotypes in the study area (Table 3). GT1, which was not known previously, was collected at sites 2, 6 and 8. It had the shortest ITS1 found so far in any *Ectocarpus* isolate (302–306 bp). GT2 was common at site 3, but also present at the most southern locality, site 10. It did not match well with any published ITS1; the best blast hit was an isolate from Puerto Deseado, Patagonia, Argentina (91% identity). However, when we sequenced ITS1 of CCAP1310/40 from site 9 (for which only the Rubisco spacer sequence had hitherto been available; U38736) its variable part showed 98% identity with that of our isolates of GT2. GT3 was collected once (at site 6); its variable part of ITS1 presented 85% identity to that of GT2. It also resembled (93% identity) that of the isolate from Argentina mentioned above, which again gave the best blast hit. Additional hits with reasonably high levels of identity were strains from New Zealand (U38761; 88%) and Isla Robinson Crusoe, Chile (U38763; 84%). GT5 was present from site 3 to site 10. It showed 97–98% identity to sequences of *E. fasciculatus* from Isla Robinson Crusoe and from France. GT6 was the only *Ectocarpus* collected at site 7, and was also common at site 8. It matched published sequences of *E. siliculosus sensu stricto* (98–99% identity). GT7, obtained from a single isolate from site 5, was not known previously; the most similar sequence available was from a *Kuckuckia* from Isla Robinson Crusoe, but it showed only 75% identity. In culture, the isolate of GT7 developed phaeophycean hairs which are characteristic for *Kuckuckia* and absent in *Ectocarpus* (Hamel, 1939). As the ITS1 of GT7 had the same length as that of *E. siliculosus*, all isolates of this ITS1 length were examined for the presence of hairs, but they were only observed in the isolate from site 5. ITS1 of two isolates of this ITS1 length from site 7 and three from site 8 were sequenced and their ITS1 matched that of *E. siliculosus*. We concluded that all isolates with an ITS1 length of 714–716 bp, apart from the one from site 5, belonged to *E. siliculosus*. GT8 was isolated only from site 7; it was highly similar (95% identity of the variable ITS1 part) to strains from Valdivia, Southern Chile, and from the salt-polluted German Werra river. Because of its long ITS1 GT8 was tentatively classified as *E. crouaniorum* despite the fact that it shared only 90% sequence identity with *E. crouaniorum* individuals from Western Europe. Phylogenetic analyses (see end of Results section) confirmed this classification. GT9 presented a double band with lengths that corresponded to *E. siliculosus* and *E. crouaniorum*. Isolates of these two species have been shown to produce hybrids, but such hybrids were unable to form meiospores (Peters *et al.*, 2010). Two strains of this putative hybrid genotype were isolated from site 7, where they co-occurred with *E. siliculosus* and *E. crouaniorum*.

The concatenated, alignable sequences from ITS1, ITS2, Rubisco spacer and *cox*3 (Table 2) had a length of 1533 bp. The different calculation methods used to generate phylogenetic trees gave comparable topologies and, consequently, only one is shown (Fig. 4). The three isolates of *Kuckuckia,* including the isolate of GT7 from site 5, formed a sister group to *Ectocarpus*, which was split into two major lineages. The first consisted of *E. fasciculatus* and an additional genotype from Europe, and the second comprised the remaining isolates. Within this latter clade, there were four subclades with strong and one subclade with moderate (> 80%) statistical support, but the hierarchy among the subclades varied and none of the hierarchies had strong statistical support in any analysis. In all trees, the genome strain clustered strongly with GT1. It was separated from *E. siliculosus*, *E. crouaniorum*, the reference strain from New Zealand and a clade formed by the genotypes 2 and 3. Strain Ec608 from site 7 (GT8), which had been tentatively classified with *E. crouaniorum* based on variable ITS1 sequences, strongly clustered with the reference strains of *E. crouaniorum* from Western Europe.

Discussion

Our study revealed a genetically diverse *Ectocarpus* flora in Peru and northern Chile (Fig. 5). The three species recognized in Western Europe were all present, including the recently reinstated *E. crouaniorum* (Peters *et al.*, 2010).

Table 3 Genotypes (GTs) and internal transcribed spacer 1 (ITS1) length types (LTs) of *Ectocarpus/Kuckuckia* from Peru and northern Chile and results of blast against sequences from previous studies

GT	Designation/ species	Field morphology[1]	ITS1 length (bp)[2]	Length of PCR product[3]	LT[3]	Examples	Sites in Peru[4]	Sites in Chile[4]	Elsewhere[5]	Best hits in blast of variable part of ITS1[6]	Identity according to blast
1	GT1	Minute dark spots on hosts	302–6	526–30	1	Ec157	2	6, 8	–	No significant hit	
2	GT2	Large thalli (Fig. 4)	344–52	568–76	2	Ec298	3	9, 10	Pto. Deseado (Argentina)	U38762	91%
3	GT3	Minute dark spot on host	347	571	2	Ec156	–	6	Pto. Deseado (Argentina)	U38762	93%
4	GT4 (the genome-sequenced *Ectocarpus*)	Small to large thalli (Figs 2, 3)	362	586	2	Ec721	1–3	5	–	AJ550048	100%
5	*E. fasciculatus* Harvey	Minute dark spots on hosts or saxicolous	431–3	655–7	3	Ec310	3	5, 8, 10	Isla Robinson Crusoe (Chile); Roscoff (France)	U38780 U38781	98% 97%
6	*E. siliculosus* (Dillwyn) Lyngbye	From thin felt on rock or invertebrates to large drifting thalli	714–6	938	4	Ec147	–	7, 8	Cosmopolitan	U38755 U38757	99% 98%
7	*Kuckuckia* sp.	Minute thallus on rope	714	938	4	Ec160	–	5	Isla Robinson Crusoe (Chile)	U38825	75%
8	*E. crouaniorum* Thuret	Minute thalli on rock and invertebrates	855	1079	5	Ec608	–	8	Valdivia (Chile), Werra (Germany); Roscoff (France)	U38772–4, FN564442	95% 90%
9	Putative hybrid sil + cro	Minute thalli on rock and invertebrates	nd	nd	4+5	Ec513	–	8	Roscoff (France)	–	–

sil, *Ectocarpus siliculosus*; cro, *Ectocarpus crouaniorum*; nd, no data; –, not applicable.
[1]For strains isolated from filtered water or developed in culture on inoculated substratum the field morphology is unknown.
[2]ITS1 : start atcattaCCGA, end GTTGTAaaacttt (Stache-Crain *et al.*, 1997).
[3]Compare with Fig. 3.
[4]Compare with Fig. 1.
[5]Origin of strains giving best blast hits.
[6]See Materials and Methods section for limits of this DNA region.

In addition there was a *Kuckuckia* genotype and four genotypes of *Ectocarpus* which have not been recorded outside the study area, and for which we can only propose provisional designations, that is genotypes (GTs) 1–4. Identification of strains based on ITS1 length (Peters *et al.*, 2010) proved insufficient in the present study as several genetically different isolates had similar ITS1 lengths. Sequencing of more (if not all) samples or PCR with genotype-specific primers will be required in similar studies in future.

Kuckuckia had been reported previously from Isla Robinson Crusoe but, based on sequence data, our isolate was different from both that strain and from six other previously sequenced isolates from Europe and South Africa which form two lineages within *Kuckuckia* (Stache-Crain *et al.*, 1997). Our new strain from northern Chile adds a third lineage. Based on culture studies, all *Kuckuckia* were merged into a single species, *Kuckuckia spinosa* (Kützing) Kuckuck (Pedersen, 1989); the molecular data and our new isolate suggest that the systematics of the genus may need to be reassessed.

Our data suggest a separation of the study area into two parts. The northern half, extending from Ancón, central Peru to Pisagua, northern Chile (sites 1–6), was dominated by the genotype of the genome strain (GT4) which represented 61% of the strains isolated in this half but was absent further south. The southern half, ranging from Antofagasta to Quintay (sites 7–10), was dominated by *E. siliculosus*, *E. crouaniorum* and their hybrids. They represented 65% of the samples in the southern half but were missing in the north. This apparent split may reflect adaptation to different oceanographic environments (see the next paragraph), but could also be an artefact of the different sampling methods: in the northern region we collected at the end of summer from drift or subtidal material, while in the southern region we collected in spring and summer in the intertidal zone and from spores in the water column. The sampling zone and season certainly influence which species of *Ectocarpus* are encountered; for instance, in Brittany, *E. crouaniorum* occurs only in the high intertidal zone and macrothalli are seasonal in spring and early summer (Peters *et al.*, 2010). More complete sampling would be required for an accurate quantification of the occurrence of the different genotypes in the area.

Isolates similar to the genome strain were the northernmost samples (Table 1). They represented two-thirds of the isolates from Peru. The distribution area of this genotype is characterized by continuous upwelling of cool water (16–20°C at the sea surface) irregularly interrupted by El Niño events which may result in several weeks of > 10°C higher sea surface temperatures as a result of the southward incursion of warm waters (Peters & Breeman, 1993). To date no *Ectocarpus* has been collected from similar low latitudes. It remains to be determined whether this genotype extends even further north, perhaps up to the deep-water kelp forests around Galápagos where its frequent host, *Desmarestia*, is present (Graham *et al.*, 2007). We have not found GT4

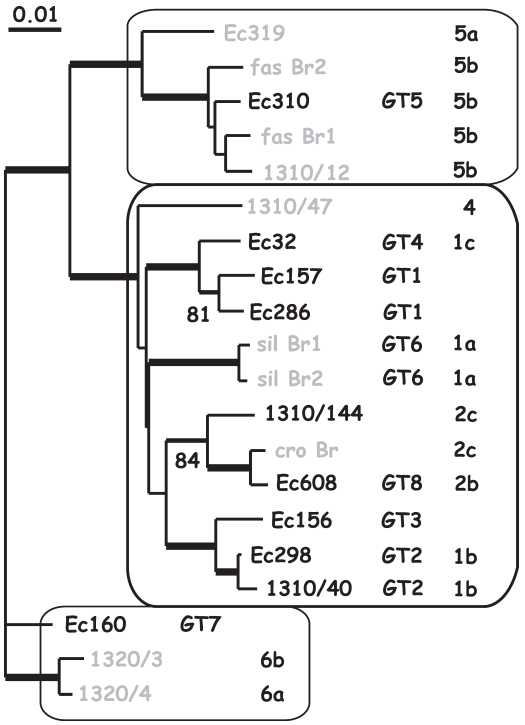

Fig. 4 Molecular phylogeny of *Ectocarpus* and *Kuckuckia* inferred from genetic distance analysis based on concatenated alignable sequences from internal transcribed spacer 1 (ITS1), ITS2, Rubisco spacer and cytochrome-c oxidase subunit 3 (*cox*3). Maximum likelihood and maximum parsimony analyses gave similar results. Thick lines represent branches having 100% bootstrap support in all analyses. Minimum bootstrap values (in one of the three different analyses) for two branches with moderate support are provided under the respective branch. All other branches had < 60% bootstrap support. Labels of the isolates from the study area, including the genome strain Ec32, are in black, and those of the reference strains in grey. The three boxes illustrate the principal split into *Kuckuckia* (bottom), *Ectocarpus* section *fasciculati* (top) and *Ectocarpus* section *siliculosi* (centre). cro Br, fas Br and sil Br are reference strains of *E. crouaniorum*, *E. fasciculatus* and *E. siliculosus sensu stricto*, respectively, from France (Peters *et al.*, 2010). Names starting with '1310' and '1320' are Culture Collection of Algae and Protozoa (CCAP) strain designations for *Ectocarpus* and *Kuckuckia*, respectively; those with 'Ec' are accessions at the *Ectocarpus* strain collection held at Roscoff, France. Genotypes obtained in the present study (GT1–8) are provided to the right of the tree, as well as the corresponding lineage numbers (1a–6b) in Stache-Crain *et al.* (1997). *Ectocarpus siliculosus sensu stricto* was in our analyses only represented by isolates from France because individuals from Chile had highly similar ITS1 sequences (98–99% identity in blast; Table 3) and sequences of additional markers were not generated for them. Ec319 represents lineage 5a in Stache-Crain *et al.* (1997) instead of strain CCAP1310/100 originally employed in Stache-Crain *et al.* (1997), for which *cox*3 is not yet available. Accessions of sequences utilized are provided in Table 2. The units in the scale bar are substitutions per site.

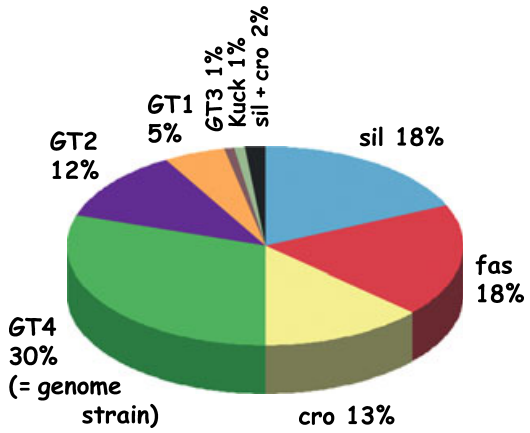

Fig. 5 Diversity of *Ectocarpus* and *Kuckuckia* in Peru and northern Chile (n = 120 samples). sil, *Ectocarpus siliculosus*; fas, *Ectocarpus fasciculatus*; cro, *Ectocarpus crouaniorum*; sil + cro, putative hybrid between *E. siliculosus* and *E. crouaniorum*; Kuck, *Kuckuckia* sp.; GT1–4, other genotypes, including that of the genome strain (GT4). For details see Table 3.

south of Arica. Apart from the two individuals from Arica, which were collected *in situ* at 3–5 m depth, all samples of this genotype were from drift material, and their original habitat and depth distribution are not known. We have also only limited data on the seasonality of this genotype. In November 1988 it was present as a minute epiphyte or endophyte in *Desmarestia peruviana*. Our collections of macroscopic thalli of GT4 in 2006 were from late summer. The Museo de Historia Natural, Lima, houses seven herbarium specimens which are thalli of *Ectocarpus*, collected by César Acleto close to Lima between September and February from 1964 to 1976. If we assume that they belong to the same genotype, macroscopic thalli appear to be present in the field from September to March.

Our phylogenetic analyses, based on the genetic markers used in Stache-Crain *et al.* (1997) plus *cox*3, confirmed most lineages obtained in that pioneer study. However, the lineages 2a, 2d and 3 in Stache-Crain *et al.* (1997) were not represented in our taxon set. Our analyses confirmed that GT4 groups within the *Ectocarpus* subclade '*siliculosi*' (Fig. 4). In a previous analysis involving fewer taxa (Peters *et al.*, 2010), it had clustered with Stache-Crain *et al.*'s (1997) lineage 1a (*E.siliculosus sensu stricto*). The addition of more South American strains led to its separation from that lineage. The genome strain was also genetically distant from *E. crouaniorum* and from an *Ectocarpus* from New Zealand. The latter had been referred to as *E. siliculosus* in previous studies (Stache, 1989; Müller, 1991; Peters *et al.*, 2004b). However, phylogenetic analyses (Stache-Crain *et al.*, 1997; and the present paper) as well as crossing studies (Stache, 1989) indicated that it is separated from *E. siliculosus sensu stricto*.

Three other genotypes of *Ectocarpus* found in Peru and Chile did not cluster with any of the recognized species. GT1 was found in both parts of the study area. It formed minute dark spots on *Macrocystis* and *Lessonia*, was new to science, and in phylogenetic analyses clustered with GT4. Cross-fertility of these two GTs has not yet been examined. Thalli of GT2 were large (Fig. 2c) and saxicolous or epiphytic on different red and brown algae, such as *Gracilaria* and *Desmarestia*. GT2 co-occurred with GT4 at site 3. In phylogenetic analyses, GT2 did not cluster with GT4. A cross between the genome strain and a member of GT2 from site 8 produced viable hybrids with weakly growing erect thalli which did not form meiosporangia (A.F. Peters, unpublished data). Together these results suggest that GT2 and GT4 belong to different species. GT3, found once as a minute epiphyte at an exposed site, was previously unknown. In phylogenetic analyses it was separated from GT4 but it clustered with GT2; its variable part of ITS1 showed 84–93% identity with those of previous isolates from Atlantic South America, New Zealand and Isla Robinson Crusoe (Stache-Crain *et al.*, 1997). Possibly GT2 and GT3 belong to a species that is more widely distributed in the Southern Hemisphere. GT3 has not so far been involved in cross-fertility tests.

In summary, the genome-sequenced *Ectocarpus* appears to be genetically separated from all genotypes with recognized names and also from all but one (GT1) of the genotypes endemic to Pacific South America or the Southern Hemisphere; it could therefore be regarded as a further species of *Ectocarpus*.

A proportion of the seaweeds that are reported from Peru and Chile also occur in the northeast Pacific (Santelices, 1989); this is the case for *Ectocarpus acutus* Setchell et Gardner reported in Chile from Coquimbo and Cartagena (Santelices, 1989; Ramírez & Santelices, 1991). It is possible that the lineages GT1+4 and 2+3 belong to this or another one of the species of *Ectocarpus* described from the Pacific coast of North America (Setchell & Gardner, 1922, 1925) or northeast Asia (Yoshida *et al.*, 1995; Yoshida, 1998). So far, four isolates of *Ectocarpus* from this vast region have been sequenced. Strains from San Francisco and Santa Barbara (California, USA) and from Kanagawa, Enoshima Prefecture, central Japan, belonged to *E. siliculosus sensu stricto*, while a strain from Akkeshi, northeast Hokkaido, Northern Japan, by contrast, was more closely related to GT4 (Stache-Crain *et al.*, 1997; Tanaka *et al.*, 2010). The Akkeshi strain was nevertheless fully compatible in crosses with *E. siliculosus sensu stricto*, but it has not been tested against GT4 (Müller & Kawai, 1991). More comprehensive sampling in the North Pacific may be helpful for the taxonomic revision of the genus and the nomenclature of Pacific South American *Ectocarpus*.

Another aim of our work was to find strains that were sexually compatible with the genome strain. Seven out of our 36 new strains of GT4 produced unilocular sporangia in culture; female gametophytes obtained from meiospores

of two sporophytes from sites 2 and 5 were used for crosses with the genome strain. In both combinations the zygotes developed into sporophytes capable of meiosis, and one of these sporophytes was selected for further experiments leading to the production of the genetic map of the *Ectocarpus* genome (Heesch *et al.*, 2010).

Acknowledgements

We thank César Acleto for permission to study specimens in the phycological herbarium at the Museo de Historia Natural, Lima, and Matt Hall for advice during the molecular experiments. A.F.P. was supported by a travel grant from Université Pierre et Marie Curie for the collections and by a Ray Lankester Investigatorship by the Marine Biological Association of the United Kingdom for the molecular work. J.B. was supported by a Darwin Initiative Pre-project grant (Darwin Reference: EIDPR35). Collections and isolations in Chile were supported by FONDAP 1501 0001 (CONICYT) awarded to the Center for Advanced Studies in Ecology & Biodiversity (CASEB) Program 7. Additional support to J.A.C. was provided by an ICA grant.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Notes S1 Details of molecular strain characterization, PCR and sequencing.

Table S1 Primers used for PCR and sequencing.

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