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Culture collection of freshwater microalgae from the Azores archipelago: resource for taxonomic and phycoprospecting research

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Résumé – Collection de cultures de microalgues d'eau douce de l'archipel des Açores : ressource pour la recherche taxonomique et la phycoprospection. Ces dernières années, un grand intérêt a été porté sur le potentiel biotechnologique des microalgues, notamment du fait de leur rapide croissance et de l'identification de certaines substances synthétisées par ces organismes. L'isolation puis la culture *in vitro* d'espèces de microalgues natives sont très importantes pour les études taxonomiques et les études de conservation. Elles représentent les premières étapes des études de faisabilité pour des productions commerciales locales. Dans cette étude, 114 microalgues ont été isolées à partir d'échantillons prélevés dans 23 stations de l'île de São Miguel, Açores. Parmi celles-ci, 60 espèces ont été identifiées dont 39 Chlorophytes, 10 Ochrophytes, 6 Cyanophytes, 3 Charophytes, 1 Euglénozoaire et 1 Cryptophyte. Dix-huit de ces identifications d'espèces constituent de nouveaux enregistrements pour l'île de São Miguel.

Microalgues natives / Nouveaux reports / Isolement / Collection de cultures

Abstract – In recent years, much interest has been focused on the biotechnological potential of microalgae, mainly due to their rapid growth and the identification of several substances synthesized by these organisms. The isolation and *in vitro* cultivation of native microalgae species is very important for taxonomic and conservation studies and is the first step towards feasibility studies of local commercial productions. In this study 114 isolates of microalgae were obtained from samples collected in 23 locations of the island of São Miguel, Azores. From them, 60 species were identified comprising 39 Chlorophyta, 10 Ochrophyta, 6 Cyanophyta, 3 Charophyta, 1 Euglenozoa and 1 Cryptophyta. Eighteen of the species identified constitute new records for the Island of São Miguel.

Native microalgae / new records / isolation / culture collection

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INTRODUCTION

Photosynthetic microalgae are ubiquitous and inhabit oceans, freshwater bodies, rocks, soils and even trees (Chen, 2008). The existing number of microalgae species in the wild is still unknown with quotes reporting figures between 200 000 to several million representatives (Patil *et al.*, 2005). This high diversity results in a high biotechnological potential as microalgae are known as a source of an outstanding amount of natural products (Norton *et al.*, 1996; Pulz & Gross, 2004). The search for local species of microalgae with biotechnological interest has been increasing in recent years mainly due to their adaptation to prevailing climatic conditions (Wilkie *et al.*, 2011).

Recently the term "phycoprospecting" was introduced to explain that native microalgae species have long been naturally selected and are therefore adapted to their local regions (Wilkie *et al.*, 2011). Local species are thus evolutionarily primed for local bioresource productions (Wilkie *et al.*, 2011). Furthermore, the choice of local species minimizes the risk of environmental impact in the event of accidental release from large-scale commercial culture (Mohsenpour *et al.*, 2012). The biotechnological use of local species requires an effective selection of microalgae strains obtained through isolation and the resulting pure cultures (Duong *et al.*, 2012).

For a long time taxonomical studies on microalgae were mainly based on morphological characters however the use of molecular tools is increasing, especially for the classification of higher groups (e.g. Cavalier-Smith, 2004; Komárek *et al.*, 2014; Leliaert *et al.*, 2012), genus (e.g. Hegewald *et al.*, 2010; Bock *et al.*, 2011; Stepanek & Kociolek, 2014) and species complexes (e.g. Saunders, 2005; Abarca *et al.*, 2014) in the aim of producing a DNA librairy of life (Le Gall *et al.* 2017). The isolation and culture of microalgae are an important step in the development and application of molecular tools for the classification of algae, contributing significantly to taxonomic and biodiversity studies (Vaulot *et al.*, 2004; Hegewald *et al.*, 2013; Song *et al.*, 2014).

The Azores archipelago, relatively isolated in the mid Atlantic Ocean (36° 55' to 39° 43' N and 24° 45' to 31° 17' W), is well known for its many freshwater bodies. The Azorean freshwater algal flora has been described as species poor, dominated by species with cosmopolitan distribution (Bohlin, 1901; Bourrelly & Manguin, 1946a, 1946b). However, taxonomic studies on Azorean freshwater algae are rare and, to our knowledge, they have not before been isolated neither subject of biotechnological investigations. Due to the isolation and remoteness of the archipelago, a high degree of endemism could be expected as it has been observed on other remote islands (e.g. Falkland by Flower (2005); Hawaii by Sherwood *et al.* (2014); maritime Antarctic region by Vijver & Beyens (1997).

The present study is focused on the survey, isolation and establishment of culture collections of indigenous Azorean microalgae to build the basis for subsequent taxonomic and biotechnological studies.

MATERIALS AND METHODS

Sources of isolates

Considering, that the volcanic origin of Azorean freshwater ecosystems determines the geology of the substrate and the geomorphology of the basin which

Table 1. Sampling sites on São Miguel Island

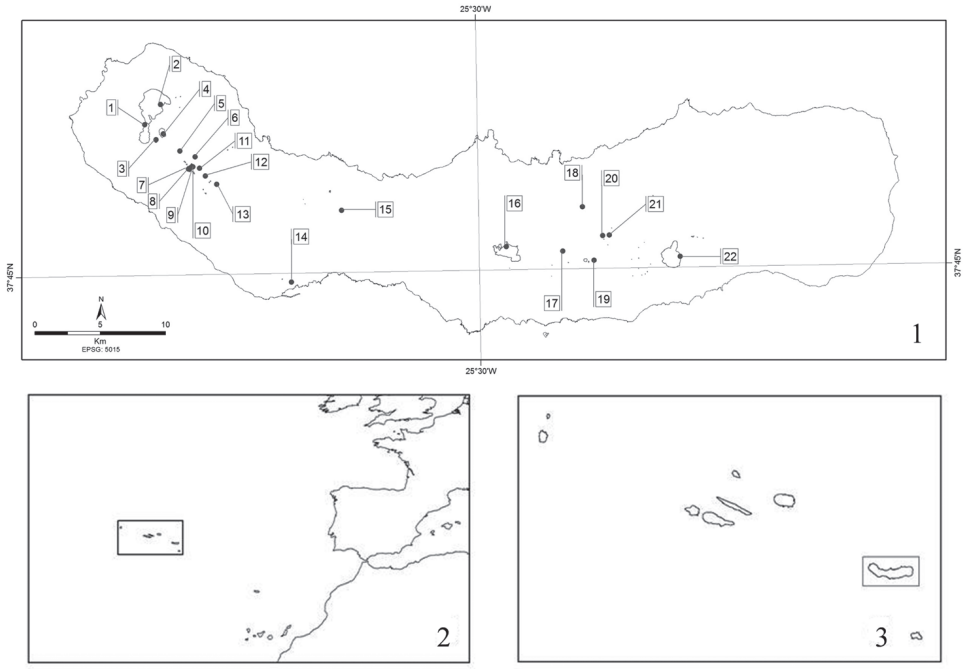
Site	*Geographic Coordinates*	*Elevation (m)*
Areeiro	37.76294444; -25.42719444	638
Azul	37.86177778; -25.62527778	259
Caldeirão Norte	37.82394444; -25.75005556	777
Caldeirão Sul	37.82319444; -25.75052778	777
Canário	37.83594444; -25.75877778	759
Canas	37.81266667; -25.72719444	588
Carvão	37.82383333; -25.74172222	687
Congro	37.75633333; -25.40011111	444
Empadadas Norte	37.82516667; -25.74819444	760
Empadadas Sul	37.82486111; -25.74769444	760
Fogo	37.76680556; -25.47625	574
Furnas	37.75808333; -25.32536111	291
Pau Pique	37.83169444; -25.74544444	702
Peixe	37.81847222; -25.73683333	631
Pico da Pedra	37.79341111; -25.61897222	159
Pico do Rei I	37.77347222; -25.38652778	591
Pico do Rei III	37.77319444; -25.39236111	599
Ponta Delgada	37.74453333; -25.66336111	34
Rasa Serra Devassa	37.82344444; -25.75158333	774
Rasa Sete Cidades	37.84405556; -25.77933333	565
Santiago	37.84794444; -25.77294444	372
São Brás	37.79327778; -25.40955556	619
Verde	37.85447222; -25.78894444	259

in turn affects the hydrological regime and the chemical properties of the lakes, water samples for microalgae isolation were collected aseptically from 23 different locations (Table 1) on the Island of São Miguel Azores (Figs 1-3), corresponding to 70% of this island lakes (according to Porteiro 2000 there are 33 lakes in the island). This sampling effort was made to guaranty that we covered the major environmental gradients occurring in the island lakes (e.g. size- from small to large; depth- from shallow to deep; altitude- 34 to 777 m; thermal regime- warm monomitic and holomitic; trophy- oligotrophic to eutrophic; pH- 5 to 9; Electric Conductivity- 30 to 150 μScm-1).

Sampling was performed in one single occasion (from June to August 2013) by taking a sample of superficial water directly from each site and storing into a cooler for transportation to the laboratory.

Culture media and conditions

Both liquid and agar culture mediums, were prepared using a commercial fertilizer developed for this purpose (see Table 2), at a concentration of 0.2 ml/L of



Figs 1-3. **1.** São Miguel Island with sampling sites. (1) Azul; (2) Verde; (3) Rasa Sete Cidades; (4) Santiago; (5) Canário; (6) Pau Pique; (7) Rasa Serra Devassa; (8) Caldeirão Norte; (9) Caldeirão Sul; (10) Empadadas; (11) Carvão; (12) Peixe; (13) Canas; (14) Ponta Delgada; (15) Pico da Pedra; (16) Fogo; (17) Areeiro; (18) São Brás; (19) Congro; (20) Pico do Rei I; (21) Pico do Rei III; (22) Furnas. **2.** Azores archipelago off the coast of mainland Portugal. **3.** Localization of the island of Sao Miguel.

Table 2. Composition of medium used for liquid and agar culture mediums

	Component	*Stock concentration (g/L)*	*Medium concentration (mol/L)*
Macronutrients	N	200	0,003
	P_2O_5	50	7,05E-05
	K_2O	40	8,49E-05
	MgO	45	0,0002
	S	60	0,0004
Micronutrients	B	0.8	1,45E-05
	Cu	0.16	5,00E-07
	Fe	1.6	5,71E-06
	Mn	0.8	2,91E-06
	Mo	0.016	3,33E-08
	Zn	0.5	1,54E-06

macronutrients and 0.2 ml/L of micronutrients. Both mediums were autoclaved at 1.21 atm. for 20 min before use.

Cultures were made with atmospheric CO_2 without extra supply of this component. Illumination was provided by 4 cool-light fluorescence lamps (18 W) to an intensity of 2500 Lux and the diurnal cycle was 12h day/12h night at a temperature of 25 ± 2°C.

Isolation and identification of microalgae

Microalgae were isolated from the samples as follows. 50 µL of sample was spread on 10 cm Petri plates containing the culture medium previously described solidified with 1.5% (w/v) of bacteriological agar. This procedure was done in triplicate for each location. The Petri plates were sealed to avoid contamination and incubated for 10 days under the conditions previously described. After the colony formation, single colonies were picked up under a magnifier (Leica Zoom 2000) and transferred to 5 mL glass test tubes with sterilized liquid medium, that were also incubated under the same conditions. The tubes were examined aseptically using optical microscopy for algal growth and to ascertain the existence of a single species in each isolate. All culture manipulations were performed on a laminar flow chamber to ensure aseptic conditions. The purity of the cultures was ensured by regular observations under a microscope. After a period of incubation, which ranged from 30 to 90 days, the isolated species were identified by optical microscopy (ZEISS AXIOIMAGE A1 with ZEISS MRc5 digital camera) using different identification keys (e.g. Huber-Pestalozzi, 1938, 1961; Ettl, 1983; Komárek & Fott, 1983; Dillard, 1981-1993; Förster, 1982; Fott, 1968; Krammer & Lange-Bertalot, 1986-2000; Komárek & Anagnostidis, 2008). Each strain was attributed a code (Figs 4-7), photographed and inserted into a database. In the event of not achieving a uni algal culture, the steps described earlier were repeated until this was obtained. The algal collection was incorporated in the herbarium Ruy Telles Palhinha of the Department of Biology of the University of the Azores (AZB) and is maintenance is assured through the European Union Interreg project REBECA (MAC/1.1a/060).

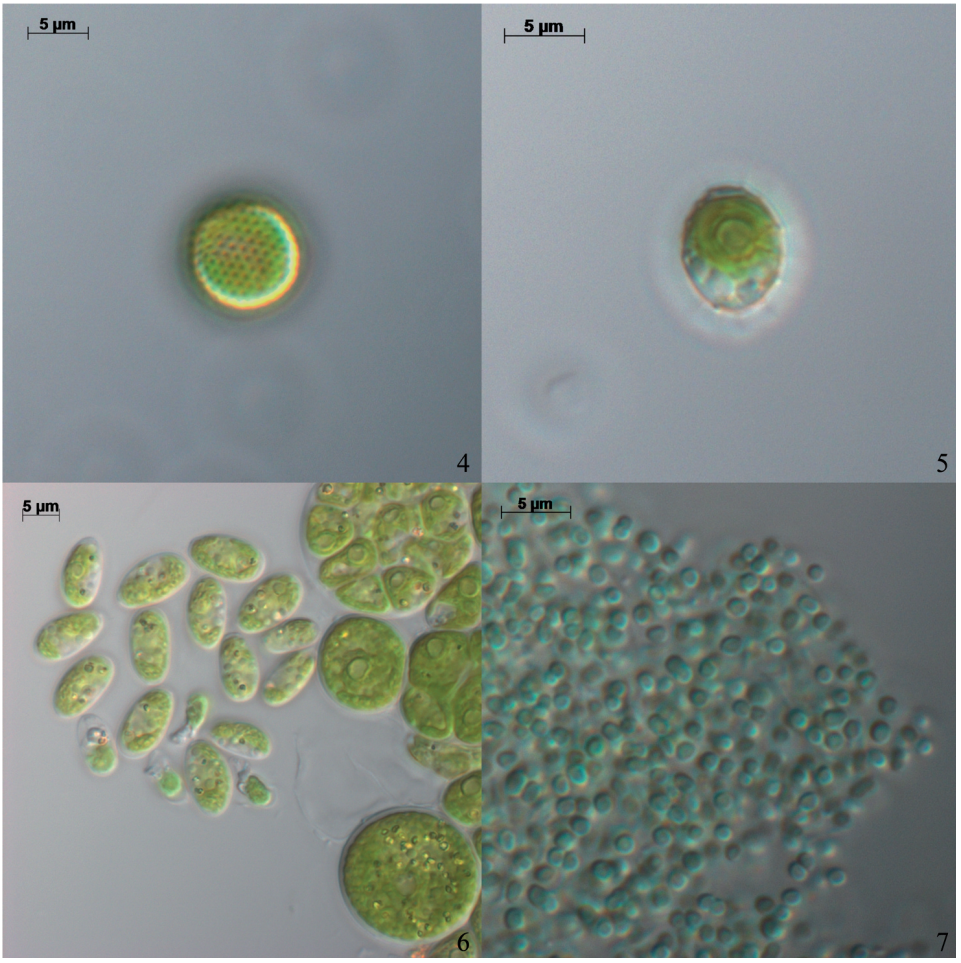
RESULTS

A total of 114 isolates were obtained from the 23 sampling sites (Table 3). The isolates corresponded to 60 species comprising 39 Chlorophyta, 10 Ochrophyta, 6 Cyanophyta, 3 Charophyta, 1 Euglenozoa and 1 Cryptophyta From these, 18 isolates constitute new records for the Island of São Miguel (see highlights in Table 3).

Most species were confined to one or two locations, but a few (e.g. *Chlorella vulgaris*, *Desmodesmus aculeolatus*, *Chlorella* sp.) were widespread in the majority of the studied lakes.

DISCUSSION

The methodology used in the present study for collection, isolation, identification and maintenance of microalgae, was successful in establishing a



Figs 4-7. **4.** *Arachnochloris* sp. **5.** *Siderocelis ornata.* **6.** *Chlorococcum* sp. **7.** *Cyanobium plancticum.*

culture collection of 114 local microalgae cultures. Isolation methodology applied in the present study used agar, following Andersen (2005) that stated this is the preferred method for isolation of coccoid and soil algae. The same methodology has been utilized in other studies, e.g. by Yue & Chen (2005) for strains of the genus *Chlorella*, by Abou-Shanab *et al.* (2011) for a total of 33 microalgae cultures, and by Abdelaziz *et al.* (2014) for over one hundred of microalgae species.

The obtained isolates demonstrated good adaptation to the commercial fertilizer culture medium utilized, which, therefore, is recommended for future studies. The isolates were mostly green algae (Chlorophyta), which is not surprising because this group is the most diverse in the plankton of Azorean lakes (Gonçalves, 2008). A prevalence of chlorophytes was also reported by Abou-Shanab *et al.* (2011) and by Abdelaziz *et al.* (2014) for other regions.

Small coccoid greens namely from the genera *Oocystis* and *Monoraphidium*, frequent and occasionally abundant in the Azorean lakes (Gonçalves, 2008), pose

Table 3. List of strains isolated

Phylum	Species	Code	Origin	Reference
Charophyta	*Cosmarium phaseolus* Brébisson ex Ralfs 1848	MIA-SMG-2013-88	Congro	INOVA, 1999
	Spirotaenia sp.	MIA-SMG-2013-21	Peixe	Bohlin, 1901; Cedercreutz, 1941
	Teilingia granulata (J. Roy & Bisset) Bourrelly 1964	MIA-SMG-2013-129	Rasa Serra Devassa	Gonçalves, 2008
Chlorophyta	*Auxenochlorella protothecoides* (Krüger) Kalina & Puncochárová 1987	MIA-SMG-2013-34	Pau Pique	**New record for the Azores**
	Bracteacoccus **sp.**	MIA-SMG-2013-8	Rasa Sete Cidades	**New record for the Azores**
	Chlamydomonas sp.	MIA-SMG-2013-9	Rasa Sete Cidades	Several (e.g. Bohlin, 1901; Cedercreutz, 1941; Johansson, 1976; Oliveira, 1989)
		MIA-SMG-2013-69	Empadadas Norte	
		MIA-SMG-2013-97	Caldeirão Sul	
		MIA-SMG-2013-109	Peixe	
		MIA-SMG-2013-119	Caldeirão Norte	
	Chlorella mirabilis V.M.Andreyeva 1973	MIA-SMG-2013-12	Ponta Delgada	**New record for the Azores**
	Chlorella sp.	MIA-SMG-2013-33	Pau Pique	Several (e.g. Gonçalves, 2008; Gonçalves *et al*., 2006)
		MIA-SMG-2013-36	Caldeirão Sul	
		MIA-SMG-2013-39	Pico do Rei 3	
		MIA-SMG-2013-52	Fogo	
		MIA-SMG-2013-63	Verde	
		MIA-SMG-2013-66	Empadadas Sul	
	Chlorella vulgaris Beyerinck [Beijerinck] 1890	MIA-SMG-2013-18	Pico da Pedra	Several (e.g. Gonçalves, 2008; Gonçalves *et al*., 2006)
		MIA-SMG-2013-20	Ponta Delgada	
		MIA-SMG-2013-70	Empadadas Norte	
		MIA-SMG-2013-74	Rasa	
		MIA-SMG-2013-79	Carvão	
		MIA-SMG-2013-90	Azul	
		MIA-SMG-2013-104	Ponta Delgada	
		MIA-SMG-2013-105	Peixe	
		MIA-SMG-2013-122	Furnas	
	Chlorococcum **sp.**	MIA-SMG-2013-19	Ponta Delgada	**New record for the Azores**
		MIA-SMG-2013-54	Canas	
		MIA-SMG-2013-125	Furnas	
		MIA-SMG-2013-134	Carvão	
	Chloromonas paradoxa Korshikov 1926	MIA-SMG-2013-77	Rasa	**New record for the Azores**
	Desmodesmus communis (E.Hegewald) E.Hegewald 2000	MIA-SMG-2013-38	Pico do Rei 3	Several (e.g. Krieger, 1931; Johansson, 1977; Gonçalves, 2008)
		MIA-SMG-2013-53	Fogo	
	Dictyosphaerium subsolitarium Van Goor 1924	MIA-SMG-2013-71	Empadadas Norte	Several (e.g. Gonçalves, 2008; Gonçalves *et al*., 2006)
	Dimorphococcus lunatus A.Braun 1855	MIA-SMG-2013-112	Peixe	Several (e.g. Gonçalves, 2008; Gonçalves *et al*., 2006)
	Gloeocystis sp.	MIA-SMG-2013-58	Canas	Gonçalves, 2008
	Haematococcus pluvialis Flotow 1844	MIA-SMG-2013-7	Ponta Delgada	Xavier *et al*., 2014
	Hariotina reticulata P.A.Dangeard 1889	MIA-SMG-2013-102	Ponta Delgada	Several (e.g. Bohlin, 1901; Cedercreutz, 1941; Gonçalves, 2008)
	Korschpalmella mucosa (Korshikov) Hindák 1988	MIA-SMG-2013-17	Pico da Pedra	**New record for the Azores**
		MIA-SMG-2013-50	Fogo	
	Leptosira **sp.**	MIA-SMG-2013-23	Empadadas Norte	**New record for the Azores**
	Monoraphidium circinale (Nygaard) Nygaard 1979	MIA-SMG-2013-67	Empadadas Sul	Several (e.g. Gonçalves, 2008; Gonçalves *et al*., 2006)
		MIA-SMG-2013-108	Peixe	
	Monoraphidium contortum (Thuret) Komárková-Legnerová 1969	MIA-SMG-2013-22	Empadadas Sul	Several (e.g. Gonçalves, 2008; Gonçalves *et al*., 2006)
		MIA-SMG-2013-43	Pico do Rei 1	
		MIA-SMG-2013-128	Rasa Serra Devassa	
		MIA-SMG-2013-132	Pico do Rei 1	
	Monoraphidium griff f ithii (Berkeley) Komárková-Legnerová 1969	MIA-SMG-2013-101	Ponta Delgada	Santos & Santana, 2009
	Monoraphidium pusillum (Printz) Komárková-Legnerová 1969	MIA-SMG-2013-41	Pico do Rei 3	Several (e.g. Gonçalves, 2008; Gonçalves *et al*., 2006)
	Mucidosphaerium pulchellum (H.C.Wood) C.Bock, Proschold & Krienitz 2011	MIA-SMG-2013-6	Empadadas Norte	Several (e.g. Bohlin, 1901; Cedercreutz, 1941; Gonçalves, 2008)
		MIA-SMG-2013-51	Fogo	
		MIA-SMG-2013-59	Canas	
		MIA-SMG-2013-91	Azul	
		MIA-SMG-2013-94	Pau Pique	
	Nephrocytium limneticum (G.M.Smith) G.M.Smith 1933	MIA-SMG-2013-111	Peixe	Gonçalves, 2008
	Oocystis lacustris Chodat 1897	MIA-SMG-2013-73	Empadadas Sul	Several (e.g. Gonçalves, 2008; Gonçalves *et al*., 2006)
		MIA-SMG-2013-87	Congro	
		MIA-SMG-2013-92	Santiago	
		MIA-SMG-2013-118	Caldeirão Norte	
		MIA-SMG-2013-123	Furnas	
	Oocystis parva West & G.S.West 1898	MIA-SMG-2013-16	Pico da Pedra	Several (e.g. Oliveira, 1989; Gonçalves, 2008)
		MIA-SMG-2013-103	Ponta Delgada	
	Oocystis rhomboidea Fott 1933	MIA-SMG-2013-82	Carvão	Several (e.g. Gonçalves, 2008; Gonçalves *et al*., 2006)
	Oocystis sp.	MIA-SMG-2013-60	Verde	Several (e.g. Gonçalves, 2008; Gonçalves *et al*., 2006)
		MIA-SMG-2013-83	Carvão	
	Oocystis submarina Lagerheim 1886	MIA-SMG-2013-78	Rasa	Oliveira, 1989; Gonçalves, 2008
	Desmodesmus aculeolatus (Reinsch) P.M.Tsarenko 2000	MIA-SMG-2013-28	Caldeirão Norte	Gonçalves, 2008
		MIA-SMG-2013-35	Caldeirão Sul	
		MIA-SMG-2013-62	Verde	
		MIA-SMG-2013-95	Pau Pique	
		MIA-SMG-2013-115	Areeiro	
		MIA-SMG-2013-117	Caldeirão Norte	
		MIA-SMG-2013-127	Rasa Serra Devassa	
	Scenedesmus armatus (R.Chodat) R.Chodat 1913	MIA-SMG-2013-42	Pico do Rei 3	Several (e.g. Cedercreutz, 1941; Oliveira, 1989; Gonçalves, 2008)
	Scenedesmus armatus **var. pseudohelveticus**	MIA-SMG-2013-45	Pico do Rei 1	**New record for the Azores**
	Scenedesmus ecornis (Ehrenberg) Chodat 1926	MIA-SMG-2013-25	Santiago	Several (e.g. Gonçalves, 2008; Gonçalves *et al*., 2006)
	Desmodesmus granulatus (West & G.S.West) Tsarenko 2000	MIA-SMG-2013-30	Canas	Azevedo *et al*., 2005

Table 3. List of strains isolated. *(continued)*

Phylum	Species	Code	Origin	Reference
	Acutodesmus obliquus (Turpin) Hegewald & Hanagata 2000	MIA-SMG-2013-14	Pico da Pedra	Cédercreutz, 1941; Gonçalves, 2008
	Desmodesmus opoliensis (P.G.Richter) E.Hegewald 2000	MIA-SMG-2013-26	Pico do Rei 3	Several (e.g. Gonçalves, 2008; Gonçalves *et al.*, 2006)
		MIA-SMG-2013-85	Canário	
	Scenedesmus semipulcher Hortobágyi 1960	MIA-SMG-2013-130	São Brás	Gonçalves *et al.*, 2005; Azevedo *et al.*, 2005
		MIA-SMG-2013-133	Pico do Rei 1	
	Scenedesmus sp.	MIA-SMG-2013-64	Verde	Several (e.g. Gonçalves, 2008; Gonçalves *et al.*, 2006)
		MIA-SMG-2013-80	Carvão	
		MIA-SMG-2013-107	Peixe	
		MIA-SMG-2013-126	Furnas	
		MIA-SMG-2013-110	Peixe	
	Desmodesmus spinosus (Chodat) E.Hegewald 2000	MIA-SMG-2013-65	Verde	Several (e.g. Krieger, 1939; Cedercreutz, 1941; Oliveira, 1989; Gonçalves, 2008)
	Siderocelis ornata (Fott) Fott 1934	MIA-SMG-2013-15	Pico da Pedra	**New record for the Azores**
	Sphaerellopsis sp.	MIA-SMG-2013-10	Rasa Sete Cidades	Several (e.g. Gonçalves, 2008; Gonçalves *et al.*, 2006)
Cryptophyta	*Cryptomonas* sp.	MIA-SMG-2013-120	Caldeirão Norte	Oliveira, 1989; Gonçalves, 2008
	Anathece minutissima (West) Komárek, Kastovsky & Jezberová 2011	MIA-SMG-2013-32	Pau Pique	Several (e.g. Gonçalves, 2008; Gonçalves *et al.*, 2006)
		MIA-SMG-2013-49	Fogo	
	Cyanobium planctonicum (G.Drews, H.Prauser & D.Uhlmann) Komárek, Kopecek & Cepák 1999	MIA-SMG-2013-37	Pico do Rei 3	**New record for the Azores**
		MIA-SMG-2013-48	Verde	
		MIA-SMG-2013-99	Caldeirão Sul	

taxonomic questions. In fact, it has been pointed out by Krienitz & Bock (2011) that the concept of genera and species within Oocystaceae remains obscure and the classification of the genus needs revision (Stoynea *et al.*, 2007). Their isolation and cultivation, as done in the present study, will allow to provide material for studies with either traditional or molecular techniques in the aim to clarify the taxonomy of these complex genera and eventually result in the description of new endemic species.

Despite the high recent interest in microalgae biotechnology, there are still many species that require research in this area. Richmond (2004) states that it is estimated that more than 50,000 species exist, but only a limited number (around 30,000), has been studied and analyzed. In view of this we recommend further phycoprospecting studies, complemented with research aimed at the evaluation of biotechnological potential of new isolates.

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