

Taxonomic investigation of *Ralfsia*-like (Ralfsiales, Phaeophyceae) taxa in the North Atlantic Ocean based on molecular and morphological data, with descriptions of *Pseudoralfsiaceae* fam. nov., *Pseudoralfsia azorica* gen. et sp. nov. and *Nuchella vesicularis* gen. et sp. nov.

Manuela I. Parente¹, Robert L. Fletcher², Filipe O. Costa^{3, 4} and Gary W. Saunders⁵

¹ CIBIO/InBio, Universidade dos Açores, Faculdade de Ciências e Tecnologia, Campus Universitário de Ponta Delgada, Rua da Mãe de Deus, 13A, 9500 Ponta Delgada, Portugal.

² University of Portsmouth, Institute of Marine Sciences, Ferry Road, Eastney, Portsmouth, Hampshire, PO4 9LY, United Kingdom.

³ CBMA – Centre of Molecular and Environmental Biology, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal.

⁴ Institute of Science and Innovation for Bio-Sustainability (IB-S), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.

⁵ Centre for Environmental & Molecular Algal Research, Department of Biology, University of New Brunswick, Fredericton, NB E3B 5A3, Canada.

CONTACT Manuela I. Parente. E-mail: nelaparente@hotmail.com

ABSTRACT

In this study we investigated *Ralfsia*-like crusts (i.e. excluding *Ralfsia sensu stricto*, *Stragularia* spp. and/or Scytosiphonaceae crustose phases) with an emphasis on the North Atlantic Ocean using molecular data (COI-5P, ITS and *rbcL*-3P) combined with morpho-anatomical comparisons of type material and contemporary specimens. Of the four species of *Ralfsia* [previously](#) reported in Europe, only *R. fungiformis* is presently recognized as belonging to *Ralfsia sensu stricto*, Ralfsiaceae, with the remaining species, viz. *R. lucida*, *R. ovata* and *R. verrucosa*, of uncertain taxonomic status. The present study revealed 11 independent genetic lineages of *Ralfsia*-like taxa, which were not assignable to any of the recognized families of the Ralfsiales. To accommodate this diversity, we propose Pseudoralfsiaceae Parente, Fletcher & G.W.Saunders *fam. nov.*, including two new genera *Pseudoralfsia* Parente, Fletcher & G.W.Saunders *gen. nov.* and *Nuchella* Parente, Fletcher & G.W.Saunders *gen. nov.* The first genus includes *Pseudoralfsia verrucosa* (Areschoug) Parente, Fletcher & G.W.Saunders *comb. nov.* as the generitype (the only species of the three remaining European species of uncertain taxonomic status assigned to *Ralfsia* that was reassessed here), *P. azorica* Parente, Fletcher & G.W.Saunders *sp. nov.* and seven undescribed genetic groups, which require additional vouchers for description. The second genus has its generitype *Nuchella vesicularis* Parente, Fletcher & G.W.Saunders *sp. nov.*, and also *N. sp.* 1MP, which was represented by a single sterile specimen from Swanage, United [Kingdom](#) and consequently not characterized. Three characteristics differentiate *Nuchella* from *Pseudoralfsia* – [the former](#) typically [with n](#)umerous vesicles, non-synchronous development of the plurangia and hair pits arising from both middle and lowers cells of the erect filaments. Species of Pseudoralfsiaceae can be distinguished from *Ralfsia sensu stricto* (Ralfsiaceae) mainly by DNA sequences and by consistently having frequent hair pits, and typically unsymmetrical thalli.

ARTICLE HISTORY

KEYWORDS brown crusts; COI-5P; DNA Barcode; *Nuchella vesicularis*; phylogenetic; *Pseudoralfsia azorica*; *Pseudoralfsia verrucosa*; Pseudoralfsiaceae; Ralfsiales; taxonomy.

Introduction

Systematics of the brown algal order Ralfsiales (Phaeophyceae), proposed by Nakamura (1972), has been considerably unstable. Long considered nomenclaturally invalid and representing an artificial grouping, it was later validated and amended by Lim *et al.* (2007) based on Rubisco large subunit gene (*rbcL*) sequence data. Within the order, these authors recognized the family Ralfsiaceae as comprising the genera *Analipus*, *Endoplura*, *Heteroralfsia* and *Ralfsia*, and proposed a new family Neoralfsiaceae to accommodate the new genus *Neoralfsia*, which was based on *Ralfsia expansa* (J.Agardh) J.Agardh. They also recognized the family Mesosporaceae, but with some hesitation probably owing to the lack of inclusion of the generitype in their analyses. Finally, they also revealed the presence of a separate clade within the order, which they named “?Ralfsia?”, that was genetically distant from members of all the other families and based on two unidentified specimens informally referred to *Ralfsia*. Subsequent molecular studies justified recognition of Mesosporaceae by including the generitype *Mesospora schmidtii* Weber-van Bosse, while at the same time providing a better understanding of several other known and novel species of *Mesospora* (Poong *et al.*, 2013, 2014, 2017). Most recently Léon-Alvaréz *et al.* (2017) proposed the new family Hapalospongidiaceae based on the generitype *Hapalospongidion gelatinosum* De A.Saunders while Parente & Saunders (2019) published a morphological and molecular evaluation of *Ralfsia sensu stricto*, Ralfsiaceae in Canada. Of the species previously

assigned to this genus, only the type species *R. fungiformis* (Gunnerus) Setchell & N.L.Gardner remained while three new species were described. Excluded from *Ralfsia sensu stricto* were *R. pacifica* Hollenberg and *R. verrucosa* (Areschoug) Areschoug. Of the four species of *Ralfsia sensu stricto* recognized by Parente & Saunders (2019), only *R. robertii* Parente & G.W.Saunders was confirmed in Europe, although European *R. fungiformis*-like specimens were not included in that study. Based on traditional taxonomic studies emphasizing morphology (excluding *Stragularia* spp. and/or scytosiphonacean crustose phases) four species of *Ralfsia* are currently recognized in European waters: *Ralfsia fungiformis* reported for Iceland; *R. verrucosa* widely reported from Iceland, Norway down to the Canary Islands, and also for the Black Sea and Mediterranean; *R. ovata* Rosenvinge reported for Iceland; *R. lucida* S.Lund reported for the Baltic Sea (Guiry & Guiry, 2018).

The aim of the present study was to investigate species-level taxonomy of *Ralfsia*-like crusts [excluding *Ralfsia sensu stricto* ([see Parente & Saunders, 2019](#)), *Stragularia* spp. and/or Scytosiphonaceae crustose phases] with an emphasis on the North Atlantic Ocean using [COI-5P](#) and [rbcL](#) to assist alpha taxonomy and place these species into a phylogenetic context.

Material and methods

Morphology

Ralfsia-like specimens (n = 75) were collected from many locations with an emphasis on the European coasts of France (Mediterranean coasts, [n = 2](#)), Norway ([n = 3](#)), Portugal [mainland ([n = 11](#)) the Azores ([n = 19](#)) and Madeira ([n = 3](#)) archipelagos], England ([n = 23](#)), with additional collections from North American coasts of Canada [Pacific([n = 7](#)) and Atlantic ([n = 5](#)) coasts] and the USA (Atlantic coast, [n = 2](#)) (Supplementary table S1). Specimens were dried (pressed or in silica gel) or preserved in 4% formaldehyde solution to serve as vouchers with subsamples

preserved in silica gel for molecular analyses (Saunders & McDevit, 2012). Specimens collected during the present survey are deposited in the Connell Memorial Herbarium at the University of New Brunswick in Fredericton and in the Herbarium Ruy Telles Palhinha at the University of the Azores in São Miguel. Type specimens of *R. confusa* Hollenberg, *R. integra* Hollenberg and *R. pacifica* were provided on loan from the U.S. National Herbarium (US) and of *R. verrucosa* from the Swedish Museum of Natural History (S). Sections were made from portions of samples using a freezing microtome (CM 1850, Leica, Heidelberg, Germany) and both sections and squash mounts of thalli were stained with 1% aniline blue in 6% 5 N HCl and mounted in 50% corn syrup with 4% formaldehyde. Observations were made and microphotographs recorded on a Leica DFC480 or Leica CH-9435 digital camera mounted on a Leica DM5000B or a Leica DM2500 light microscope, respectively (Leica, Heidelberg, Germany). Measurements of vegetative and reproductive structures are always presented as height (perpendicular to the substratum) and length/width (parallel to the substratum).

DNA extraction, amplification and sequencing

Total DNA was extracted and the mitochondrial COI-5P (DNA barcode region 5' end of the mitochondrial cytochrome *c* oxidase I gene; n = 66; 658 sites) and the nuclear ITS (internal transcribed spacer of the ribosomal cistron, whole region ITS1-5.8S-ITS2; n = 8; 919 sites) were amplified according to Saunders & McDevit (2012) and McDevit & Saunders (2017), respectively. The full fragment of *rbcL* was also amplified (n = 17; 781 sites), both to assign genetic groups based on *rbcL*-3P and to perform phylogenetic analyses. For species delineation using *rbcL*-3P only unidirectional sequence data from the reverse external primer were generated (Saunders & Moore, 2013). The primers used are recorded with each GenBank accession (Supplementary tables S1 and S2, BOLD Dataset: RGWS2EUR). For phylogenetic analyses the

rbcL was sequenced using the external primers 33F (Draisma *et al.*, 2010) and 1381R (Burrowes *et al.*, 2003), as well as the internal primers 543F (Bittner *et al.*, 2008), PRB-R1A (Kogame *et al.*, 1999) and KR2 (Lane *et al.*, 2006). PCR products were sequenced using a Big Dye terminator cycle sequencing kit v3.0 (PE Applied Biosystems, Foster City, Canada) and an ABI 3130XL sequencer.

Molecular analyses

Identification of genetic groups was performed through barcode gap analyses (based on raw p-distances) of the COI-5P (n = 66; 658 sites) and *rbcL*-3P (n = 20; 781 sites) data in BOLD and Geneious 10.2.3 (Kearse *et al.*, 2012), respectively. Phylogenetic analyses were performed using each of the two genes separately as well as a concatenated alignment (species and sequences included in the alignments are listed in Supplementary table S1); in all cases incorporating additional COI-5P and *rbcL* sequence data for other [phaeophycean](#) species available in GenBank (Supplementary table S2). [To expand our phylogenetic trees *rbcL* and COI-5P sequences of closely related orders \(Fucales, Nemodermales and Tilopteridales\) were retrieved from GenBank \(Supplementary tables S2\) and trees were rooted with members of Dictyotales \[*Dictyopteris polypodioides* \(D.C.\) J.V. Lamouroux and *Dictyota dichotoma* \(Hudson\) J.V. Lamouroux\], Sphacelariales \[*Cladostephus spongiosus* \(Hudson\) C. Agardh\] and Syringodermatales \(*Syringoderma phinneyi* E.C. Henry et D.G. Müller\) based on published phylogenies in Silberfeld *et al.* \(2010\).](#) Prior to the combined analyses, COI-5P (n = 55; 658 sites) and *rbcL* (n = 75; 1301 sites) alignments were analysed individually to test for topology congruence. The concatenated alignment of 1959 sites included 75 taxa, all of which had *rbcL* data and 55 for which COI-5P data were available (Supplementary table S2). Phylogenetic inferences were completed using RAxML (Stamatakis, 2014) in Geneious R8 to run a maximum

likelihood (ML) analysis with the model GTR+I+G, partitioned by gene and codon, with 1000 bootstrap replicates.

Results

Identification of species

The DNA barcode analyses of *Ralfsia*-like crusts (i.e. excluding *Ralfsia sensu stricto*, *Stragularia* spp. and/or Scytosiphonaceae crustose phases) revealed 11 strongly divergent genetic groups (only *Pseudoralfsia* sp._4MP and sp._5MP were closely allied with 2.27% divergence in their COI-5P sequences; Table 1). Of these 11 genetic groups, only one was assignable to a known species (discussed below), *Ralfsia verrucosa* [= *Pseudoralfsia verrucosa* (Areschoug) Parente, Fletcher & G.W.Saunders *gen. et comb. nov.*]. This species had COI-5P intraspecific divergence of 0.33% and was 10.69% divergent from its nearest neighbour *Pseudoralfsia* sp._10MP, while *rbcL*-3P divergence was 1.54% from *P.* sp._3MP (*P.* sp._10MP lacks COI-5P data) (Table 1). *Pseudoralfsia verrucosa* (n = 27) was found on the coast of Norway (n = 3), the United Kingdom (n = 10), and the North American Atlantic (n = 7) and Pacific (n = 7) coasts (Supplementary table S1).

Pseudoralfsia azorica Parente, Fletcher & G.W.Saunders *sp. nov.* was based on two samples with a single COI-5P haplotype from the Azores and 19.46% divergence from their nearest neighbour, *P.* sp._5MP (Table 1).

Owing to the close relationship for *Pseudoralfsia* sp._4MP and *P.* sp._5MP (2.27% divergence in COI-5P; Table 1), ITS data were also generated, but regrettably for only three specimens – one for *P.* sp._4MP and two for *P.* sp._5MP). Only three fixed differences were noted between *P.* sp._4MP and *P.* sp._5MP ITS sequences (two substitutions and one indel of five nucleotides), which calls into question two distinct genetic groups, but more study is needed.

Nuchella vesicularis Parente, Fletcher & G.W.Saunders *gen. et sp. nov.* (n = 11) had COI-5P intraspecific divergence of 0.91% (Table 1), ITS intraspecific divergence of 0.27% and 11.55% COI-5P divergence from its nearest-neighbour, *N. sp._1MP* (n = 1). It was also divergent for *rbcL*-3P (6.36%) from its nearest neighbour *P. verrucosa* (Table 1). The former species was found on the coasts of mainland Portugal (n = 9), Madeira (n = 1) and the United Kingdom (n = 1).

Phylogenetic analyses

Maximum likelihood analysis of the single gene analyses were largely consistent where branches had support and an analysis combining the two regions was completed. The analysis for the concatenated *rbcL* and COI-5P genes for specimens collected by us and identified as *Ralfsia*-like resolved as three distinct lineages among recognized families of the Ralfsiales – the first proposed here as the new family Pseudoralfsiaceae Parente, Fletcher & G.W.Saunders *fam. nov.*, as well as two distinct lineages among other families of Ralfsiales that will be presented in a future publication (provisionally named Ralfsiacean_GWS3 and GWS4, Fig. 1). *Pseudoralfsia* was fully supported in the concatenated alignment and included eight genetic groups, which joined *Nuchella* in the moderately supported (80% bootstrap support) new family Pseudoralfsiaceae, (Fig. 1). In total, Pseudoralfsiaceae included 11 genetic groups (*N. sp._1MP* and *P. sp._10MP* lacked *rbcL* data, and were not included in the concatenated analysis, Table 1) with three of them formally described here. The Pacific *Ralfsia* sp. C (AB250075, Lim *et al.*, 2007) joined the family and resolved within *Pseudoralfsia* (Fig. 1).

Taxonomic results

Pseudoralfsiaceae Parente, Fletcher & G.W.Saunders, *fam. nov.*

Description

Crusts circular or indefinite in outline, with or without rhizoids and with or without superimposed thalli; erect filaments straight or curved upwards (rarely curved downwards); cells with a single chloroplast lacking obvious pyrenoids; hair pits frequent, arising from the middle and or lower cells of erect filaments. Unangia sessile, or on 1, rarely 2-3 pedicel cells, on the terminal erect filaments and accompanied by 1-2 paraphyses; sori of plurangia non-adventitious, with synchronized and non-synchronized development of the plurangia; plurangia uniseriate or biseriate, intercalary on the erect filaments with one, occasionally two, sterile terminal cells.

TYPE GENUS: *Pseudoralfsia* Parente, Fletcher & G.W.Saunders *gen. nov.*

Pseudoralfsia* Parente, Fletcher & G.W.Saunders, *gen. nov.

Description

Thalli circular or indefinite in outline, with or without rhizoids and with or without superimposed thalli; erect filaments straight or curved upwards (rarely curved downwards); cells with a single chloroplast lacking obvious pyrenoids; hair pits frequent arising from the lower cells of erect filaments. Unangia, sessile or on pedicels of one, rarely 2-3 cells, on terminal erect filaments and accompanied by 1-2 paraphyses; sori of plurangia are non-adventitious with a synchronized development, plurangia uniseriate or at times biseriate, intercalary in the erect filaments with a sterile terminal cell.

TYPE SPECIES: *Pseudoralfsia verrucosa* (Areschoug) Parente, Fletcher & G.W.Saunders *comb. nov.*

ETYMOLOGY: The species of this genus have been confused with true *Ralfsia* and for that reason we assign the name ‘false *Ralfsia*’.

***Pseudoralfsia verrucosa* (Areschoug) Parente, Fletcher & G.W.Saunders *comb. nov.* (Figs 2-15)**

BASIONYM: *Cruoria verrucosa* Areschoug, *Algarum* (phycearum) minus rite cognitarum pugillus secundus. *Linnaea*. 1843: 264, pl. 9, figs 5, 6.

HETEROTYPIC SYNONYMS: *Ralfsia verrucosa* var. *lignicola* Areschoug 1847, *Ralfsia verrucosa* var. *cochlearum* Areschoug 1876.

LECTOTYPE: Swedish Museum of Natural History, A4006 (Figs 2-8).

TYPE LOCALITY: Bohuslän, Western Götaland, Sweden (Areschoug, 1843)

Observations on the lectotype (Figs 2-8; Table 2)

Crust dark brown, confluent and indefinite; surface irregular and warty without obvious external concentric zones or radial lines (Fig. 2). Crust typically strongly verrucose as a result of overgrowth of small successive lobes and spaces between lobes (Fig. 3) with individual crusts up to 284 µm thick. Underside of crusts rust-red coloured and rarely with rhizoids (Fig. 4); lower stratum mostly composed of horizontally elongated (4-11 µm x 5-32 µm; Fig. 4), occasionally quadrate (5-6 µm) cells, which give rise to assurgent, rarely descending, curved cell rows.

Assurgent curving cells rows grading into erect filaments (Fig. 4), the cells of which were variable in size from quadrate (5-9 μm) to vertically elongated (7-23 μm x 3-11 μm); cells with 1 plate-like chloroplast lacking obvious pyrenoids; hair pits frequent arising from lower cells of erect filaments (Fig. 5). Sori of unangia gelatinous, elevated, frequently in a medium-large sized patch, also in multiple small patches, but never forming continuous sori (Fig. 3). Unangia abundant, of markedly different sizes in the same sorus and between samples (Figs 6-8); subclavate or clavate, mainly sessile, but at times on 1, rarely 2, celled pedicels and terminating the erect filaments, accompanied by 1-2 paraphyses. Occasionally immature unangia start to develop early in their maturation process. Mature unangia up to 71 μm high and 37 μm in diameter; paraphyses simple, clavate, up to 13 cells (123 μm) high and up to 12 μm wide at the apex, gradually narrowing with frequently longer cells below (Figs 6-8).

Observations of specimens assigned to our genetic group (Figs 9-15; Table 2)

Crusts light to dark brown, sometimes olive in colour and with a lighter margin, circular in outline or sometimes confluent and indefinite, up to 3 cm broad; crusts typically with an irregular and warty surface without obvious external concentric zones and radial lines (Figs 2 and 9).

Crusts strongly verrucose as a result of overgrowth of small successive lobes and spaces between lobes (Fig. 10), or less verrucose or even smooth, with individual crusts up to 270 μm thick.

Underside of crusts rust-red coloured; lower stratum mostly composed of horizontally elongated (4-12 μm x 4-35 μm ; Fig. 11), occasionally quadrate (5-12 μm) cells, which give rise to assurgent, rarely descending, curved cell rows. Assurgent curving cells rows grading into erect filaments (Fig. 11), the cells of which were variable in size from quadrate (5-8 μm) to vertically elongated (6-24 μm x 4-12 μm); cells with 1 plate-like chloroplast lacking obvious pyrenoids; hair pits frequent arising from lower cells of erect filaments (Fig. 12). Sori of unangia gelatinous,

elevated, frequently in a medium-large sized patch, also in multiple small patches, but never forming continuous sori. Unangia abundant, of markedly different sizes in the same sorus and between specimens (Figs 13-15); subclavate or clavate, mainly sessile, but at times on 1, rarely 2-3, celled pedicels and terminating the erect filaments, accompanied by 1-2 paraphyses. Mature unangia 54 - 115 μm high and 21 - 38 μm in diameter; paraphyses simple, clavate, 10 - 14 cells (54 - 215 m) high in the central region of mature sori and up to 10 μm wide at the apex, gradually narrowing with frequently longer cells below (Figs 13-15).

HABITAT, DISTRIBUTION AND REPRODUCTION: Specimens were collected growing on rock, cobble, stones, molluscs and wood from high upper intertidal to shallow subtidal (to 0.25 m depth) habitats, including sheltered and exposed sites, with one sample collected from a salt march tidal stream (MS116, GWS022408, GWS030522, GWS022409, GWS007132, MS109, MS427a, MS114, MS313, GWS014106, MS117, MS113, NOR02, MS323, GWS032188, GWS031324, MS112, NOR01, MP11N, GWS014113, GWS008755, GWS005091, GWS027814, NOR03, GWS027815, GWS014465, GWS022407; Supplementary table S1). Specimens were found on European coasts (Norway and United kingdom), as well as Northeastern American coasts (Newfoundland and Labrador, New Brunswick, Nova Scotia and Massachusetts), and Northwestern American coasts (British Columbia) (Supplementary table S1). Reproductive specimens with plurangia were found in February, May and September (Supplementary table S1).

***Pseudoralfsia azorica* Parente, Fletcher & G.W.Saunders *sp. nov.* (Figs 16-21)**

Description

Crusts brown in colour, lacking a light margin, indefinite in outline (Fig. 16), up to 2 cm broad, smooth, lacking obvious external concentric zones and radial lines. Crusts thin (Fig. 17 and 19), up to 220 μm thick; cells of lower stratum mainly horizontally elongate (2.7-5.8 μm x 7.6-14.3 μm ; Fig. 18), which give rise to straight vertical cell rows (Figs 17-19). Underside of crusts rust-red and lacking rhizoids; cells of erect filaments ranging from quadrate (4.8-8.1 μm) to vertically elongated (3.6-8.1 μm x 4.1-6.6 μm) and horizontally elongated (3.9-6.3 μm x 4.9-8.8 μm); cells with one plate-like chloroplast lacking obvious pyrenoids; hair pits abundant arising from lower cells of erect filaments (Fig. 20). Sori gelatinous, non-adventitious and in multiple small sized patches rather than a single continuous sorus (Figs 19-20). Plurangia with up to 8 loci (48 μm high), uniseriate and with 1 sterile terminal cell (6.1-9.6 μm x 4.2-8.8 μm ; Fig. 21); individual loci 4.0-8.8 μm high and 4.3-7.7 μm wide.

HOLOTYPE: Specimen MD0001334c (Figs 16-21), growing on rock in the upper mid intertidal zone, collected by M. I. Parente and R. Sousa on 8 October 2009 and deposited in the Museu de História Natural de Lisboa.

TYPE LOCALITY: Caloura (37.7, -25.5), São Miguel, Azores, Portugal.

HOLOTYPE DNA BARCODE: MK972252

ETYMOLOGY: Named for its type locality in the Azores.

HABITAT, DISTRIBUTION AND REPRODUCTION: Both specimens were found growing on rock in the upper mid intertidal zone (MD0001334c, MD0001334d; Supplementary table S1).

Thus far, only collected in the Azores, São Miguel, Portugal (Supplementary table S1).

Reproductive specimen with plurangia was found in October (Supplementary table S1).

Nuchella* Parente, Fletcher & G.W.Saunders, *gen. nov.

Description

Crusts circular in outline, typically lacking superimposed thalli; sometimes with rhizoids present; erect filaments slightly curving upward (rarely curving downward); cells with a single chloroplast lacking obvious pyrenoids and typically with numerous vesicles; hair pits frequent, arising from both lower and middle cells of the erect filaments. Plurangia imbedded in concave, non-adventitious sori with non-synchronous development. Plurangia uniseriate or biseriate, intercalary in erect filaments with one, occasionally two, sterile terminal cells.

TYPE SPECIES: *Nuchella vesicularis* Parente, Fletcher & G.W.Saunders, *sp. nov.*

ETYMOLOGY: Named in honour of Dr. Ana Cristina Costa (affectionately known as Nucha), a valued friend of the senior author (M.I.P.), in recognition of her constant encouragement.

***Nuchella vesicularis* Parente, Fletcher & G.W.Saunders, *sp. nov.* (Figs 22-27)**

Description

Crusts light to medium brown in colour sometimes with a lighter margin, circular in outline (Fig. 22), up to three cm broad; surface regular and smooth, typically with radial lines and sometimes with concentric zones. Typically lacking superimposed thalli; 120-417 μm in thickness (Fig. 23); underside of crusts rust-red coloured, occasionally with multicellular rhizoids (Fig. 24). Lower region of stratum composed mostly of 4 to 6 layers of horizontally elongated (2.4-15.5 μm x 9.1-27.5 μm) and occasionally quadrate (3.7-15.5 μm) cells, which give rise to assurgent (Fig. 23), rarely descending, slightly curving cell rows; middle cells of erect filaments of variable sizes from small quadrate (7-10 μm), often vertically elongated (6.4-25.6 μm x 3-14.8 μm) and at times horizontally elongated (6-15 μm x 10-17 μm); cells of the apical region of the erect filaments often horizontally elongated (4.2-10.6 μm x 5.0-11.6 μm), quadrate (5-11.6 μm) or, also vertically elongated (5.6-10.6 μm x 5-8.6 μm); cells with one plate-like chloroplast lacking obvious pyrenoids and typically with several intracellular vesicles (Fig. 25); hair pits frequent arising from both lower and middle cells of the erect filaments. Sori gelatinous, in multiple, small-sized patches rather than in a single continuous sorus, imbedded, concave and non-adventitious with non-synchronous development of the plurangia (Figs 26-27); plurangia up to 14 loci, uniseriate, sometimes biseriate with 1 or 2 sterile terminal cells (5.0-16.0 μm x 2.5-5.8 μm ; Fig. 27); individual loci 2.1-7.2 μm high and 2.5-5.7 μm wide; unangia unknown.

HOLOTYPE: Specimen MD0001687 (Figs 22, 25-27), growing on rock in the mid intertidal zone, collected by M. I. Parente and R. Sousa on 24 July 2010 and deposited in Museu de História Natural de Lisboa.

TYPE LOCALITY: Gaia, São Felix da Marinha, Portugal.

HOLOTYPE DNA BARCODE: MK972260

ETYMOLOGY: Named on the basis of the presence of several vesicles in each cell.

HABITAT, DISTRIBUTION AND REPRODUCTION: Specimens were collected growing on rock in the mid intertidal zone in mainland Portugal, Madeira and the United Kingdom (MP13N, MD0001675, MD0001660, PG071357, MD0001681, MD0001657, MD0001687, MD0001682, MPFL241; MD0001684, MPFL243, MP31N; Supplementary table S1). Reproductive specimen with plurangia was found in July (Supplementary table S1).

Discussion

The present study, which emphasized North Atlantic and notably European collections revealed hidden diversity in *Ralfsia*-like samples. Based on molecular analyses and morpho-anatomical comparisons to type material, we propose to transfer a monophyletic subset of *Ralfsia*-like species (except Ralfsiacean_GWS3 sp._1MP and Ralfsiacean_GWS4 sp._1GWS unresolved among the Ralfsiales complex of families; Fig. 1) to the [moderately supported](#) new family Pseudoralfsiaceae, Ralfsiales. This family is assigned two genera, *Nuchella* and *Pseudoralfsia*, that do not resolve within the currently recognized families (viz. Hapalospongiaceae, Mesosporaceae, Neoralfsiaceae and Ralfsiaceae; Fig. 1). Species of Pseudoralfsiaceae are morphologically similar to species of *Ralfsia sensu stricto*, Ralfsiaceae, but can be distinguished by having frequent hair pits, typically unsymmetrical thalli and, in particular, by their molecular data. Indeed, the deep genetic differences support the recognition of separate families ([see Fig. 1](#)). León-Alvarez *et al.* (2017) similarly considered sequence data of primary importance in distinguishing the Hapalospongiaceae from the Mesosporaceae, which lack robust

distinguishing morphological or anatomical features. Similar decisions were implemented by Hind *et al.* (2016) regarding the subfamilies Corallinoideae and Neogoniolithoideae and, more recently, by Caragnano *et al.* (2018) regarding the subfamily Chamberlainoideae, Corallinales. The data presented here support placement of *P. verrucosa*, *P. azorica*, *N. vesicularis* and eight additional informal genetic groups within Pseudoralfsiaceae.

The genetic group that we assigned to *Pseudoralfsia verrucosa* was distinct from all of the other genetic groups studied here with vegetative construction common to that in the lectotype of *R. verrucosa*. In both, there is a build-up of several small successive lobes resulting in thick verrucose and convoluted crusts typically lacking a symmetrical shape (see Fig. 2 and 9). This development distinguishes *P. verrucosa* from all the other Ralfsiales described to date, as well as all of the genetically identified taxa uncovered in the current manuscript. We are thus confident that this genetic group is correctly assigned to *Ralfsia verrucosa*. Besides, the lectotype of *R. verrucosa* and our samples varied with respect to the maximum dimensions of their mature unangia (71 μm x 37 μm and 54 - 115 μm x 21 - 38 μm , respectively; Table 2) and maximum length of their paraphyses (123 μm and 54 - 215 μm , respectively), the size of paraphyses and unangia of the lectotype are within the size range of our samples. Our collections of *P. verrucosa* have a widespread distribution (Pacific and Atlantic North America coasts, United Kingdom and Norway) including one specimen in close geographic proximity (less than 100 km distant in a straight line in Verdens Ende, Vestfold, Skagerrak coast of Norway) to the type locality (Bohuslän, Western Götaland, Sweden). This lends support to our morpho-anatomical observations in assigning our genetic group to this morphospecies.

Based on the present results and Parente & Saunders (2019) regarding identification conflicts within crustose species of the Ralfsiales, and the morphological variation displayed among specimens of *R. verrucosa* (discussed above), all previous reports of *R. verrucosa* should

be confirmed by sequence data.

Pseudoralfsia azorica displayed considerable COI-5P interspecific divergence from other species (19.46%) consistent or greater than that displayed between species and genera of the Ralfsiaceae, as well as between other phaeophycean species and genera (e.g. Lim *et al.*, 2007; McDevit & Saunders, 2009; Poong *et al.*, 2014; León-Alvarez *et al.*, 2017; Parente & Saunders, 2019). It differs morpho-anatomically from *P. verrucosa* by having thinner thalli, straight erect filaments (not curving) and in having smaller cell sizes (Table 2). *Pseudoralfsia azorica* differs morpho-anatomically from *Ralfsia tenebris* Parente & G.W.Saunders in bearing frequent hairs often arising from lower cells of erect filaments, in having much thinner thalli, brown colour, and different habitat preferences (found in the middle or upper littoral on rock surfaces). This species also differs from *Ralfsia confusa* Hollenberg by having brown colour and sori in multiple small sized patches. The holotype of *R. confusa* is a light brown crust, mostly with single and continuous sorus situated in the central part of the crust. As *P. azorica* was based on only two collections (Supplementary table S1), we eagerly await additional information regarding its morphological variation, seasonality, ecological and geographical ranges.

Nuchella was proposed to accommodate the generitype *N. vesicularis* sp. nov. and an informal genetic group provisionally named *Nuchella* sp._1MP, which was represented by a single sterile specimen. *Nuchella vesicularis* is both genetically distinct (Table 1) and morpho-anatomically different from *Pseudoralfsia* spp. and other crustose Ralfsiales in having embedded and concave, non-adventitious plurangia sori with non-synchronous development, as well as consistently having numerous vesicles in the cells. *Nuchella* is tentatively included in Pseudoralfsiaceae until its evolutionary affiliation can be better resolved (Fig. 1).

Acknowledgments

Many thanks to previous and current members of the Saunders Laboratory for technical assistance and for valued advice and helpful discussions. We thank all the collectors listed in Supplementary table S1 for their critical involvement in this project. Thanks also to Ana Ferreira, Elisabete Dias, Ricardo Camarinho, Maximilian Müller and Sophia Griese for laboratory help, Ana C. Costa, Mónica Moura and Armindo Rodrigues for making available laboratory equipment and Ana C. Costa also for helpful comments. Thanks are also due to Alvin K. Chan, K. Dixon, D. McDevit and T. Moore for generating some of the sequence data. We gratefully acknowledge the trustees of the U.S. National Herbarium (US) and Swedish Museum of Natural History for organizing loans of type material and providing helpful taxonomic information. We also thank Michael Wynne for assistance in interpreting historical taxonomic literature and Craig Schneider and António Frias Martins for assistance with Latin.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was funded by FEDER funds through the Programa Operacional Factores de Competitividade – COMPETE and by national funds through FCT – Foundation for Science and Technology in the scope of the MACROBIOMOL project (ref. PTDC/MAR/114613/2009) and under the UID/BIA/50027/2013 and POCI-01-0145-FEDER-006821 programmes. Work completed in the Saunders Laboratory was supported by the Canadian Barcode of Life Network from Genome Canada in association with the Ontario Genomics Institute, the Natural Sciences and Engineering Research Council of Canada, as well as other sponsors listed at

www.boldsystems.org, with infrastructure support from the Canada Foundation for Innovation and New Brunswick Innovation Foundation.

Supplementary information

Supplementary table S1. [Specimens](#) included in the present study with collection information and GenBank accession numbers. Reproductive specimens are indicated with ^a and ^b (for plurangia and unangia respectively), and accession numbers of the sequences used in the concatenated alignment are marked in bold type.

Supplementary table S2. [Sequences](#) retrieved from GenBank with collection information, publication and accession numbers. [^a, ^b, ^c, ^d used to distinguish samples of the same species.](#)

Author contributions

M.I. Parente: original concept, drafting and editing manuscript, sample descriptions; molecular data analyses and interpretation; R.L. Fletcher: original concept, sample descriptions, editing manuscript; F.O. Costa: original concept, molecular data analyses, editing manuscript; G.W. Saunders: original concept, drafting, editing manuscript, molecular data analyses, sample descriptions.

ORCID Manuela I. Parente <https://orcid.org/0000-0003-0204-7155>

References

Areschoug, J.E. (1843). Algarum (phycearum) minus rite cognitarum pugillus secundus. *Linnaea*, **17**: 257-269, pl. IX.

- Bittner, L., Payri, C.E., Couloux, A., Cruaud, C., Reviers, B. de. & Rousseau, F. (2008). Molecular phylogeny of the Dictyotales and their position within the brown algae, based on nuclear, plastidial and mitochondrial sequence data. *Molecular Phylogenetics and Evolution*, **49**: 211-226.
- Burrowes, R., Rousseau, F., Müller, D.G. & Reviers, B. de (2003). Taxonomic placement of *Microzonia* (Phaeophyceae) in Syringodermatales based on *rbcL* and 28S nrDNA sequences. *Cryptogamie Algologie*, **24**: 63-73.
- Caragnano, A., Foetisch, A., Maneveldt, G.W., Millet, L., Liu, L.-C., Lin, S.-M., Rodondi, G. & Payri, C.E. (2018). Revision of Corallinaceae (Corallinales, Rhodophyta): recognizing *Dawsoniolithon* gen. nov., *Parvicellularium* gen. nov. and Chamberlainoideae subfam. nov. containing *Chamberlainium* gen. nov. and *Pneophyllum*. *Journal of Phycology*, **54**: 391-409.
- Draisma, S.G.A., Prud'homme Van Reine, W.F. & Kawai, H. (2010). A revised classification of the Sphacelariales (Phaeophyceae) inferred from a *psbC* and *rbcL* based phylogeny. *European Journal of Phycology*, **45**(3): 308-326.
- Guiry, M.D. & Guiry, G.M. (2018). Algaebase. World-wide electronic publication, National University of Ireland, Galway (Online). Available from <http://www.algaebase.org> (accessed 18 May 2018).
- Hind, K.R., Gabrielsen, P.W., Jensen, C.P. & Martone, P.T. (2016). *Crusticorallina* gen. nov., a nongeniculate genus in the subfamily Corallinoideae (Corallinales, Rhodophyta). *Journal of Phycology*, **52**(6): 929-941.
- Kain, J.M., Buchanan, J., Boo, S.M. & Lee, K.M. (2010). *Colpomenia bullosa* crust masquerading as *Ralfsia verrucosa* (Phaeophyceae) in southeast Australia. *Phycologia*, **49**(6): 617-627.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P. & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, **28**(12): 1647-1649.

Kogame, K., Horiguchi, T. & Masuda, M. (1999). Phylogeny of the order Scytosiphonales (Phaeophyceae) based on DNA sequences of *rbcL*, partial *rbcS*, and partial LSU nrDNA. *Phycologia*, **38**(6): 496-502.

Lane, C.E., Mayes, C., Druehl, L.D. & Saunders, G.W. (2006). A multi-gene molecular investigation of the kelp (Laminariales, Phaeophyceae) supports substantial taxonomic reorganization. *Journal of Phycology*, **42**: 493-512.

León-Alvarez, D., Reyes-Gómez, V.P., Wynne, M.J., Ponce-Márquez, M.E. & Quiróz-González, N. (2017). Morphological and molecular characterization of *Hapalospongidion gelatinosum*, Hapalospongidiaceae fam. nov. (Ralfsiales, Phaeophyceae) from Mexico. *Botanica Marina*, **60**(5): 567-581.

Lim, P.-E., Sakaguchi, M., Hanyuda, T., Kogame, K., Phang, S.-M. & Kawai, H. (2007). Molecular phylogeny of crustose brown algae (Ralfsiales, Phaeophyceae) inferred from *rbcL* sequences resulting in the proposal for Neoralfsiaceae fam. nov. *Phycologia*, **46**: 456-466.

McDevit, D.C. & Saunders, G.W. (2009). On the utility of DNA barcoding for species differentiation among brown macroalgae (Phaeophyceae) including a novel extraction protocol. *Phycological Research*, **57**(2): 131-141.

McDevit, D.C. & Saunders, G.W. (2017). A molecular investigation of Canadian Scytosiphonaceae (Phaeophyceae) including descriptions of *Planosiphon* gen. nov. and *Scytosiphon promiscuus* sp. nov. *Botany*, **95**(7): 653-671.

Nakamura, Y. (1972). A proposal on the classification of the Phaeophyta. In *Contributions to the Systematics of Benthic Marine Algae of the North Pacific* (Abbott, I.A. & Kurogi, M., editors), 147-155. *Japanese Society of Phycology*. Kobe.

Parente, M.I. & Saunders, G.W. (in press). A molecular survey of *Ralfsia* sensu stricto (Ralfsiales, Phaeophyceae) in Canada uncovers three new species: *R. robertii* sp. nov., *R. tenebris* sp. nov. and *R. unimaculata* sp. nov. *Botany* (doi.org/10.1139/cjb-2018-0138).

Poong, S.W., Lim, P.E., Phang, S.M., Gerung, G.S. & Kawai, H. (2013). *Mesospora elongata* sp. nov. (Ralfsiales, Phaeophyceae), a new crustose brown algal species from the Indo-Pacific region. *Phycologia*, **52**(1): 74-81.

Poong, S.W., Lim, P.E., Phang, S.M., Sunarpi, H., West, J.A. & Kawai, H. (2014). A molecular-assisted floristic survey of crustose brown algae (Phaeophyceae) from Malaysia and Lombok Island, Indonesia based on *rbcL* and partial *cox1* genes. *Journal of Applied Phycology*, **26**(2): 1231-1242.

Poong, S.W., Lim, P.E., Phang, S.M., Sunarpi, H., West, J.A., Miller, K.A., Nelson, W. & Kawai, H. (2017). Two new species of *Mesospora* (Ralfsiales, Phaeophyceae) from the subtropical Indo-Pacific region. *Phycologia*, **56**(5): 487-498.

Saunders, G.W. & McDevit, D.C. (2012). Methods for DNA barcoding photosynthetic protists emphasizing the macroalgae and diatoms. *Methods in Molecular Biology*, **858**: 207-222.

Saunders, G.W. & Moore, T.E. (2013). Refinements for the amplification and sequencing of red algal DNA barcode and RedToL phylogenetic markers: a summary of current primers, profiles and strategies. *Algae*, **28**(1): 31-43.

[Silberfeld, T., Leigh, J. W., Verbruggen, H., Cruaud, C., De Reviers, B., & Rousseau, F. \(2010\). A multi-locus time-calibrated phylogeny of the brown algae \(Heterokonta, Ochrophyta,](#)

[Phaeophyceae\): Investigating the evolutionary nature of the “brown algal crown radiation”.](#)

[Molecular phylogenetics and evolution, 56\(2\): 659-674.](#)

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analyses and post-analysis of large phylogenies. *Bioinformatics*, open access.

Table 1. Intra- and interspecific variation in COI-5P and *rbcL*-3P for species of Pseudoralfsiaceae.

Species	COI-5P			<i>rbcL</i> -3P				
	n	MID (%)	NN	DNN (%)	n	MID (%)	NN	DNN (%)
<i>Nuchella</i>								
<i>N. vesicularis</i>	11	0.91	<i>N. sp._1MP</i>	11.55	1	N/A	<i>P. verrucosa</i>	6.86
<i>N. sp._1MP</i>	1	N/A	<i>N. vesicularis</i>	11.55	-	-	-	-
<i>Pseudoralfsia</i>								
<i>P. azorica</i>	2	0	<i>P. sp._5MP</i>	19.46	1	N/A	<i>P. verrucosa</i>	7.00
<i>P. sp. C</i>	-	-	-	-	1 ^a	N/A	<i>P. sp._3MP</i>	2.80
<i>P. sp._10MP</i>	1	N/A	<i>P. sp._3MP</i>	8.31	-	-	-	-
<i>P. sp._1MP</i>	12	0.54	<i>P. sp._3MP</i>	7.41	1	N/A	<i>P. sp._3MP</i>	0.70
<i>P. sp._2MP</i>	5	0.80	<i>P. sp._3MP</i>	4.91	3	0	<i>P. sp._3MP</i>	0.70

<i>P. sp._3MP</i>	4	1.44	<i>P. sp._2MP</i>	4.91	2	0	<i>P. sp._2MP</i>	0.70
<i>P. sp._4MP</i>	6	0.15	<i>P. sp._5MP</i>	2.27	1	N/A	<i>P. sp._5MP</i>	0.14
<i>P. sp._5MP</i>	3	0	<i>P. sp._4MP</i>	2.27	1	N/A	<i>P. sp._4MP</i>	0.14
<i>P. verrucosa</i>	21	0.33	<i>P. sp._10MP</i>	10.69	10 ^b	0	<i>P. sp._3MP</i>	1.54

Note: MID, maximum intraspecific divergence; NN, nearest neighbour; DNN, distance to nearest neighbour.

^a This sequence only comprises 587 sites (AB250075, Lim *et al.*, 2007) belonging to the *rbcL*-3P region.

^b Includes the sequences AB250074 (Lim *et al.*, 2007, from Norway, Bergen), GU014707, GU014708 (Kain *et al.*, 2010; both from the Isle of Man).

Table 2. Comparison of lectotype to our genetically verified collections assigned to *Pseudoralfsia verrucosa* (measurements in μm).

	Lectotype	Genetically verified collections
Colour	Dark brown	Light to dark brown and sometimes olive
Colour margin	Not different from the rest	Sometimes lighter
Outline	Confluent and indefinite	Circular or sometimes confluent and indefinite
Surface	Very irregular and warty, with many small successive lobes	Very irregular and warty, with many small successive lobes, or less verrucose or even smooth
Thickness of individual lobes	284	270
Cells of the lower stratum	Quadrangle 5-6 or horizontally elongated 4-11 x 5-32	Quadrangle 5-12 or horizontally elongated 4-12 x 4-35
Rhizoids	Rare	Not observed
Cells of erect filaments	Quadrangle 5-9 to vertically elongated 7-23 x 3-11	Quadrangle 5-8 to vertically elongated 6-24 x 4-12
Unangia, maximum height	67 x 37	115 x 33

and maximum diameter

Pedicel cells (number)	0-1 (2 rare)	0-1 (2-3 rare)
Paraphyses maximum number of cells (length) and maximum cell diameter	13 (123) and 12	14 (215) and 10

Figure legends

Fig. 1. Maximum likelihood (RAxML) results for the concatenated *rbcL* and COI-5P alignment. Support values are ML bootstrap values (branches lacking values had <50% support); the scale bar indicates substitutions per site. Bold type indicates the type species of *Pseudoralfsia* and *Nuchella*. [a, b, c, d used to distinguish samples of the same species \(Supplementary table S2\).](#)

Figs 2-8. *Pseudoralfsia verrucosa* (Areschoug) Parente, Fletcher & G.W.Saunders *comb. nov.*, observations of the lectotype (*Ralfsia verrucosa*, A4006). **Fig. 2.** Image of a portion of the thallus. **Fig. 3.** Radial-vertical section showing successive small lobes. **Fig. 4.** Radial-vertical section showing basal cells and one rhizoid (arrow) and erect filaments curving upward. **Fig. 5.** Radial-vertical section showing one hair pit and erect filaments curving upward. **Fig. 6.** Unangia and associated paraphyses. **Fig. 7.** Sessile unangia associated with two paraphyses. **Fig. 8.** Unangia and associated

paraphyses. Scale bars: Fig. 2, 0.3 cm; Fig. 3, 200 μm ; Fig. 4, 20 μm Fig. 5, 50 μm ; Figs 6-8, 20 μm .

Figs 9-15. *Pseudoralfsia verrucosa* (Areschoug) Parente, Fletcher & G.W.Saunders *comb. nov.*, based on specimens genetically assigned to this species. **Fig. 9.** Image of a crust growing in the intertidal excised from rock (MS109). **Fig. 10.** Radial-vertical section showing successive small lobes (GWS014113). **Fig. 11.** Radial-vertical section showing basal cells and erect filaments curving upward (GWS014113). **Fig. 12.** Radial-vertical section showing a hair pit (GWS014106). **Fig. 13.** Unangia and associated paraphyses (MS109). **Fig. 14.** Unangium on a three-celled pedicel (arrow) associated with two paraphyses (arrowheads, MS109). **Fig. 15.** Unangia and associated paraphyses (MS313). Scale bars: Fig. 9, 1 cm; Fig. 10, 100 μm ; Figs 11-12, 10 μm ; Fig. 13, 60 μm ; Figs 14-15, 50 μm .

Figs 16-21. Morpho-anatomy of *Pseudoralfsia azorica* Parente, Fletcher & G.W.Saunders *sp. nov.* **Fig. 16.** Image of the holotype (MD0001334c), growing in the upper region of the mid intertidal on rock. **Fig. 17.** Radial-vertical section showing vertical cell rows (not curved) of erect filaments (MD0001334c). **Fig. 18.** Radial-vertical section showing basal cells (MD0001334c). **Fig. 19.** Two slightly elevated sori, separated by two erect filaments (arrow; MD0001334c). **Fig. 20.** Radial-vertical section showing immature sori separated by erect filaments (arrows) and a hair pit (arrowhead; MD0001334c). **Fig. 21.** Plurangia with single sterile terminal cells

(MD0001334c). Scale bars: Fig. 16, 0.5 cm; Figs 17-18, 20 μm ; Fig. 19, 50 μm ; Figs 20-21, 20 μm .

Figs 22-27. Morpho-anatomy of *Nuchella vesicularis* Parente, Fletcher & G.W.Saunders *sp. nov.* **Fig. 22.** Image of the holotype (MD0001687), growing in the intertidal on rock. **Fig. 23.** Radial-vertical section showing slightly curved cell rows (MD0001682). **Fig. 24.** Radial-vertical section showing multicellular rhizoids (MD0001681). **Fig. 25.** Upper cells of erect filaments filled with many intracellular vesicles (MD0001687). **Fig. 26.** Radial-vertical section showing a non-adventitious sorus (arrow; MD0001687). **Fig. 27.** Detail of a sorus with uniseriate and biseriate plurangia (arrows) capped by one or two sterile terminal cells (arrowheads; MD0001687). Scale bars: Fig. 22, 0.5 cm; Fig. 23, 100 μm ; Fig. 24, 50 μm ; Fig. 25, 20 μm ; Fig. 26, 50 μm ; Fig. 27, 20 μm .