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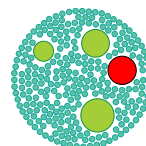
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ABSTRACT

Cystoseira sensu lato (s.l.) – encompassing the genera *Cystoseira* sensu stricto (s.s.), *Ericaria* and *Gongolaria* – is a diverse group of forest-forming brown macroalgae endemic to the warm-temperate North-east Atlantic. These algae have immense biogeographic and ecological significance and have been experiencing recent regional declines. Most *Cystoseira* s.l. display important morphological plasticity and can be confused with similar species. Therefore, species boundaries, geographic ranges and phylogenetic affinities remain imprecise for most. In the face of persistent taxonomic difficulties, several authors underlined the necessity for new molecular-based approaches, but studies so far lacked representativity, resolution and standardization. To fill in these gaps, in this study we sequenced a comprehensive collection of *Cystoseira* s.l. spanning its entire North-east Atlantic range for a ~1200 bp cox1 barcode, and sequenced selected individuals representing major genetic entities for a few additional plastid markers. Phylogeographic, phylogenetic and species delimitation methods revealed 27 Molecular Operational Taxonomic Units, including unaccounted cryptic diversity, and elucidated with unprecedented resolution species compositions and phylogenetic relationships within each genus. Some entities within the lineages *Cystoseira compressa/humilis*, *Ericaria brachycarpa/crinita*, *E. selaginoides* and tophulose *Gongolaria*, as well as among free-living algae, conflicted with a priori taxonomic assignments, and required the redefinition, reinstatement and recognition of new taxa. For some, diagnostic mutations and biogeography were more useful for species identifications than morphological characters or conventional barcoding gaps. A few species showed narrow geographic ranges and others were the sole representatives of their respective lineages. Several sister-species showed Atlantic vs Mediterranean complementary ranges. Phylogenetic signal of cox1 was nevertheless insufficient to confidently determine patterns of lineage splitting in several lineages and species complexes and did not improve significantly with additional plastid markers. We discuss novel systematics and biogeography insights considering the advantages and shortcomings of the barcoding approach employed, and how this comprehensive baseline study can be expanded to address multiple questions still left unanswered.

HIGHLIGHTS

- Identification of major genetic entities of *Cystoseira* s.s., *Ericaria* and *Gongolaria*.
- A comprehensive reference cox1 barcode library for *Cystoseira* s.l.
- Updated systematics and biogeography of *Cystoseira* s.l.

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Introduction

In the North-east Atlantic, in addition to *Fucus* wracks and *Laminaria* kelps, one of the most characteristic forest-forming algae, and endemic to the area, is the polyphyletic genus *Cystoseira* sensu lato (s.l.) (Fucales, Phaeophyceae), comprising members of the genera *Cystoseira* sensu stricto (s.s.), *Ericaria* and *Gongolaria*. *Cystoseira* s.l. assemblages are diverse and abundant in the warm-temperate Mediterranean (Hereu *et al.*, 2008; Sales & Ballesteros, 2009) and Lusitanian (Elejabeitia & Afonso-Carrillo, 1993; García-Fernández & Bárbara, 2016) marine provinces (Spalding *et al.*, 2007), a few species extending this 'core' range to adjacent cold-temperate (Britain and Ireland) and subtropical (Cape Verde) transitional regions (Oliveras-Plá & Gómez-Garreta, 1989) and to the Black/Azov seas (Sadogurska *et al.*, 2021). Like kelp forests and seagrass meadows, *Cystoseira* s.l. forests provide critical habitat, nursery grounds and food to a range of associated species, increasing the biological, structural and trophic complexity where they occur (Cheminée *et al.*, 2013; Thiriet *et al.*, 2016; Costa *et al.*, 2018). *Cystoseira* spp. s.l. are very sensitive to water quality and vulnerable to a range of local (e.g. pollution, eutrophication, overgrazing) and global (e.g. climatic change) stressors (Iveša *et al.*, 2016; Mancuso *et al.*, 2018; Boudouresque *et al.*, 2020), with severe regional declines documented in Macaronesia, the Mediterranean and the Black Sea (Minicheva *et al.*, 2013; Thibaut *et al.*, 2015; Valdazo *et al.*, 2017).

As a genus, *Cystoseira* s.l. is relatively easy to recognize but many species are difficult to identify even by trained phycologists. Similar to so many other seaweed groups, most species exhibit substantial phenotypic plasticity and most can be easily confused with similar species (e.g. tophulose *Gongolaria*, all authors, personal observations). Morphological identification often requires whole individuals, including the holdfast, cauloid and reproductive structures. In some cases, distinguishing features can be ambiguous and different authors give conflicting accounts (e.g. Sellam *et al.*, 2017). The number of infraspecific taxa and synonyms (Guiry & Guiry, 2021), and the regularity of taxonomic updates (e.g. re-instatement of taxa, reclassification, synonymy) demonstrate the problems with this group (Berov *et al.*, 2015; Bouafif *et al.*, 2016; Sellam *et al.*, 2017; Orellana *et al.*, 2019; Serio & Furnari, 2021). In addition, many geographic regions (e.g. African coast, eastern Mediterranean) remain insufficiently investigated, and a few species have very superficial descriptions and/or have been recorded only a handful of times (e.g. *C. senegalensis* P.J.L.Dangeard). Herbarium specimens could help fill knowledge gaps but they can be more challenging to identify than living specimens, while providing less information regarding potentially relevant habitat and/or morphological (e.g. colour, iridescence) features. In general, and for the abovementioned reasons, species boundaries and geographic ranges remain imprecise for many species, and the validity of a few taxa requires verification.

The implications of taxonomic uncertainty are multiple and far-reaching. Inadequate baseline information shifts focus to higher taxonomic ranks, which are easier to identify and manage. This conservative approach was followed in The Annexe II of the Barcelona Convention for the Protection of the Marine Environment and the Coastal Region of the Mediterranean, which lists most Mediterranean *Cystoseira* s.l., except for *C. compressa* (Esper) Gerloff & Nizamuddin, as endangered/threatened, without naming, as in other taxa (e.g. seagrasses, *Sargassum*), individual species (Verlaque *et al.*, 2019). Taxonomic aggregation and systematic misidentification confounds species distributions and estimates of regional diversity and endemism. It may also mask community shifts and obscure relevant species-level variation in ecological traits, potentially compromising monitoring and conservation efforts (Dulvy *et al.*, 2000; Tellier *et al.*, 2011*a*; Ensing *et al.*, 2013; Aubry *et al.*, 2017). Given the persistent taxonomic difficulties, several authors underlined the necessity for new molecular-based approaches (Reviers *et al.*, 2007; Coll *et al.*, 2010). DNA-assisted identifications are less affected by the level of taxonomic expertise and intra-specific variation, and have recurrently detected instances of misidentification, cryptic diversity, over-splitting, or simply misclassification, among macroalgae (Lindstrom, 2008; Vieira *et al.*, 2014; Neiva *et al.*, 2017).

The first comprehensive gene-based phylogenetic study of the Sargassaceae showed that *Cystoseira* was, amongst other genera, polyphyletic (Draisma *et al.*, 2010, but see also earlier studies such as Rousseau & De Reviers, 1999). Several genera were resurrected to accommodate unrelated clades, including *Stephanocystis* Trevisan (*ca.* seven North Pacific species), *Polycladia* Montagne (three Indian Ocean species) and *Sirophysalis* Kützing (one tropical Indo-West-Pacific species). Three clades endemic to the North-east Atlantic and the Mediterranean were provisionally retained within *Cystoseira*, presumably because incomplete taxon sampling and overlapping ranges complicated unambiguous clade assignment of many species. Orellana *et al.* (2019) eventually resurrected the genera *Carpodesmia* Greville 1830 and *Treptacantha* Kützing 1843 to accommodate two of these clades, but shortly after Molinari & Guiry (2020) showed that the genera *Ericaria* Stackhouse 1809 and *Gongolaria* Boehmer 1760 had nomenclatural priority over these names. Several recent studies have built on Draisma *et al.* (2010) to clarify species relationships and boundaries (Rožić

et al., 2012; Orellana *et al.*, 2019; Sousa *et al.*, 2019*a*; Jódar-Pérez *et al.*, 2020; Mulas *et al.*, 2020; Sadogurska *et al.*, 2021). These studies, based on 23S, mt23S-tRNA Val spacer, *nad*1, *psb*A and *cox*1, brought significant new insights, but have been limited to some extent by the poor resolution within species complexes (i.e. related morpho-species were not discriminated in gene trees), limited taxonomic/geographic scope (i.e. focused on a few selected taxa and/or regions), and in some lineages by poor taxonomic/geographic replication. This latter point may have overlooked implications, as misidentification of representative species 'types' and unaccounted cryptic diversity remain a real possibility.

Genetic barcoding is an increasingly popular approach in diversity inventories that can overcome some of the abovementioned limitations. Barcoding makes use of standardized DNA regions (i.e. barcodes) to catalogue biodiversity, offering a simple, fast and cost-effective way of sorting large numbers of specimens/sequences into species-like units (Schindel & Miller, 2005). Standardization has the clear benefit of allowing the calibration of taxonomic identifications and levels of divergence (intra-, interspecific) across studies. When multiple specimens from disparate locations are analysed, barcoding data can more readily detect instances of taxonomic conflict and reveal phylogeographic patterns, which are particularly useful when phylogenetic resolution is low and when species identities, boundaries and broad-scale distributions are imperfectly known (McDevit & Saunders, 2010; Radulovici *et al.*, 2010; Bartolo *et al.*, 2020). In this context, several species delimitation methods have been developed specifically for use with single-locus DNA barcodes (Puillandre *et al.*, 2012; Zhang *et al.*, 2013). Reliance on a single, uniparentally inherited, introgression-prone, organelle marker has its pitfalls (Collins & Cruickshank, 2013), but such baseline data are still useful to guide more sophisticated and expensive studies using multilocus or genomic data.

About 40 Atlantic/Mediterranean endemic species of *Cystoseira* s.l. are currently listed in AlgaeBase, excluding putative fossils and species mentioned only in pre-1900 literature (Guiry & Guiry, 2021, accessed 01–04-2021). Of these, about nine are considered essentially Atlantic, three occur in both the Atlantic and the Mediterranean, and the remaining ~25 are considered Mediterranean endemics (or predominantly). In the present study, we aimed to barcode a taxonomically and biogeographically diverse collection of samples to clarify several related aspects regarding the diversity, systematics and biogeography of *Cystoseira* s.l. Our main objectives were to: (1) identify/delimit major genetic entities within *Cystoseira* s.l. throughout its entire range, integrating species delimitation methods (SDMs) with geographic and morphological data, including potentially unaccounted cryptic taxa, and pinpoint major conflicts with morphology-based taxonomic (and biogeographic) literature, with particular focus on North-east Atlantic and western Mediterranean assemblages where main species are tentatively mapped; (2) develop a voucher-backed *cox*1 barcode library for future reference and explore the potential for a global divergence threshold to be used to delimit species; (3) assess, for species with greater biogeographic representation, the utility of *cox*1 for phylogeographic purposes; and (4) determine with greater resolution the species compositions and phylogenetic affinities within *Cystoseira* sensu stricto, *Ericaria* and *Gongolaria*.

Materials and methods

Sampling, DNA extraction and cox1 sequencing

To avoid genetic assignments based on single-individual 'types' and maximize chance discovery of cryptic/oversplit species, considerable effort was made to analyse a geographically diverse collection of as many morpho-species of *Cystoseira* s.l. as possible, including dubious or unassignable specimens. These were collected throughout the whole North-east Atlantic and Mediterranean range (i.e. from Azores to Israel and Cape Verde to British Isles) between 2017–2020, with the exception of a few older samples already present in the laboratory. We also obtained samples of appropriate outgroup genera (Draisma *et al.*, 2010). Identifications were worked based on classic textbooks (Gómez-Garreta *et al.*, 2001; Cormaci *et al.*, 2012) and publications of local experts. Tissue samples were preserved dehydrated in silica-gel. Genomic DNA was extracted using the Nucleospin® Plant II kit (Macherey-Nagel Duren, Germany), following the manufacturer protocol. DNA was diluted 1:100 for PCR reactions, except for recalcitrant samples for which dilution was individually adapted. All samples were amplified and sequenced for the mitochondrial barcode *cox*1 (= COI) gene (Saunders & McDevit, 2012), after confirming its highest resolution to discriminate *Cystoseira* s.l. species, when compared with other organelle markers (e.g. nad1, *cox*3, *rbc*L, 23S, mt23S-tRNA Val intergenic spacer, and others, original sequence data in Silberfeld *et al.*, 2010; Sousa *et al.*, 2019*a*). To increase resolution, and make full use of *cox*1 data available from GenBank (https://www.ncbi.nlm.nih.gov/genbank/), this gene was amplified as two overlapping fragments targeting a 1255 nt (34 nt of overlap) concatenated sequence (Supplementary table S1). Amplicons were sequenced in an ABI PRISM 3130xl automated capillary sequencer (Applied Biosystems) at CCMAR, Portugal. Sequences were aligned, proofread and concatenated in Geneious Prime 2020 (http://www.geneious.com).

Barcoding, species delimitation and distributions

Preliminary ML phylogenetic analyses confirmed the existence of three well-defined, evolutionarily unrelated, genera within *Cystoseira* s.l.: *Cystoseira* s.s. (related to *Stephanocystis* and other genera), *Ericaria* (related to *Bifurcaria*) and *Gongolaria* (Supplementary fig. S1). Therefore, after establishing species compositions and appropriate outgroups, each genus was analysed separately. Sequence divergence within lineages (i.e. major tree branches, often including shallow species complexes) was typically very low, and phylogenetic trees showed very little power to clarify species boundaries at this level (Supplementary fig. S1). Therefore, and to make use of potential diagnostic mutations and phylogeographic signal, genealogic relationships and diversity were illustrated using haplotype networks (instead of tree-based methods, see Collins & Cruickshank, 2013), as commonly used in phylogeographic studies. The established Statistical Parsimony TCS algorithm was used to produce haplotype networks for each genus in PopART (http://popart. otago.ac.nz), using only individuals for which the two overlapping *cox*1 fragments were complete.

To reduce subjectivity in the face of taxonomic uncertainty, low divergence and obvious genetic/taxonomic conflicts, delimitation of Molecular Operational Taxonomic Units (MOTUs, pragmatic proxies for candidate species) was explored with four species delimitation methods – jMOTU, ABGD, PTP and GMYC. jMOTU (Jones *et al.*, 2011) implements a distance-based clustering method that uses a straightforward criterion – maximum (absolute) number of mutations allowed within MOTUs – that is particularly appropriate for shallow species complexes. For each genus, thresholds for species delimitation were defined based on the observed haplotype clustering and continuity, and match with a priori taxonomic identifications. Another similarity-based or 'MOTU-picking' method, Automatic Barcode Gap Discovery (ABGD, Puillandre *et al.*, 2012), was run for each genus on the dedicated website (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html). Following exploratory runs, and based on morphotaxa and haplogroup delimitations, values of P (prior Intraspecific divergence) and X (relative gap width) in ABDG analyses were set to $0.001 < P < 0.006$ and $X = 0.007$ for *Cystoseira* s.s., $0.001 < P < 0.005$ and $X = 0.006$ for *Ericaria* and $0.001 < P < 0.002$ and $X = 0.003$ for *Gongolaria*. Model-based species delimitation methods, which make use of the evolutionary information contained in phylogenetic trees, included Poisson Tree Processes (PTP, Zhang *et al.*, 2013) and Generalized Mixed Yule Coalescent (GMYC, Fujisawa & Barraclough, 2013). These methods can discriminate between speciation and population divergence on rooted phylogenetic (PTP) or ultrametric (GMYC) trees The *cox*1 alignments were trimmed to unique sequences (i.e. to one sequence per haplotype). Best nucleotide substitution models (3 substitution schemes) were determined in jModelTest2 (Darriba *et al.*, 2012), using ML optimized tree for likelihood calculations and Best base tree searches. Best-fit models were selected using the Akaike information criterion. For PTP, maximum likelihood (ML) trees were reconstructed with RAxML v1 in the web-server https://raxml-ng.vital-it.ch/#/ (Kozlov *et al.*, 2019) using 100 bootstraps to calculate nodal support. Bayesian trees were reconstructed in MrBayes 3.26 (Ronquist *et al.*, 2012). Two parallel Metropolis-coupled Markov chain Monte Carlo searches, each with four chains (3 'heated'), were run for 2×10^6 generations, sampling trees and parameters every 200 generations (20 000 trees). Run length sufficiency was confirmed by inspecting the average standard deviation of split frequencies between runs (ASDSF < 0.02) and cold chains Log-likelihood stationarity. Based on the latter, 2×10^5 generations (1000 trees each run) were discarded as burn-in. The remaining 18 000 trees sampled were used to produce 50% majority-rule consensus trees and to calculate branch posterior probabilities. All PTP analyses were performed at http://species.h-its.org/ptp/ for 10^5 generations, selecting the best ML trees and appropriate outgroups and leaving the remaining options as default. For GMYC, ultrametric trees were reconstructed with Beast v1.10 (Suchard *et al.*, 2018). To reduce potential issues associated with prior uncertainty and poor model selection, distinct ultrametric trees were reconstructed under strict (fixed) and relaxed molecular clocks in combination with Constant Growth and Yule Coalescent tree priors. Markov Chain Monte Carlo analyses were run for 10^7 generations, starting from random trees and sampling every 1000 generations. Tracer v1.7 (Rambaut *et al.*, 2018) was used to inspect run results and confirm acceptable sample sizes (ESS> 200). Maximum clade credibility trees and posterior probability for the nodes were calculated with Beast's TreeAnnotator, after discarding 2000 trees (out of 10 000) as burn-in. Single threshold GMYC species delimitation analyses were run in R (R Core Team, 2021) for the distinct ultrametric trees using the package *Splits*.

Each MOTU considered was mapped using all useful sequences available, including complete and partial sequences and previously published *cox*1 (= COI) sequences available from GenBank (64 sequences available, see Supplementary table S2). Whenever required, systematic and nomenclatural updates were made integrating genetic data with morphological and geographic evidence (including of types), and available names.

For each genus, pair-wise divergence within and between MOTUs was estimated in MEGA v7 (Kumar *et al.*, 2016) using Kimura's two-parameter (K2P, allowing different transition/transversions rates) sequence distances. Barcoding gaps for species delimitation were investigated by comparing the degree of overlap of intra- and inter-specific K2P distances between individual sequences. Finally, to evaluate the usefulness of *cox*1 for phylogeographic studies, haplotype variation for the MOTUs better represented in the final data-set was mapped and haplotypic and nucleotide diversity was estimated with DnaSP v6 (Rozas *et al.*, 2017).

Phylogenetic analyses

A smaller collection of samples comprising one or two (in this case the most morphologically distinct) representatives of each MOTU recovered was further sequenced for fragments of the mitochondrial *nad*1, *cox*3 and the chloroplast *psa*A genes. These markers were amplified and sequenced as single fragments (Supplementary table S1).

Phylogenetic analyses were conducted separately for each genus using the appropriate outgroups, including Pacific *Stephanocystis* (*Cystoseira* s.s.) and Atlantic *Bifurcaria* R.Ross (*Ericaria*); the closest relative to *Gongolaria* remains unclear (previous studies and our own data, see Supplementary fig. S1). This

genus, however, is composed by two well-defined clades (very divergent, potentially deserving genus status) that were used as reciprocal outgroups. For each genus and gene, nucleotide substitution models were compared and selected with jModelTest v2 as above. Bayesian analyses were run as for PTP species delimitation analyses above but specifying best substitution models for each individual gene partition and using longer runs (25×10^6 generations, 75 000 saved trees) to reconstruct the consensus trees. ML analyses were run on the IQ-TREE webserver (Trifinopoulos *et al.*, 2016), with nodal support estimated with 1000 ultrafast bootstraps. Trees were rooted with the appropriate outgroups and edited in FigTree *v*.1.4.3 (http://tree.bio.ed.ac.uk/software/fig tree/).

Results

MOTUs delimitation, geographic distributions and taxonomic correspondence

A total of 288 *cox*1 barcodes were obtained for around 30 morpho-species (excluding varieties) of *Cystoseira* s.l. collected from Azores to Israel and from Cape Verde to Ireland (Fig. 1). Most taxa, with very few exceptions, were sampled from at least two regions. Each *cox*1 alignment – for *Cystoseira* s.s., *Ericaria* and *Gongolaria* – was composed by (1) 1193 base-pair long sequences corresponding to the two concatenated *cox*1 fragments
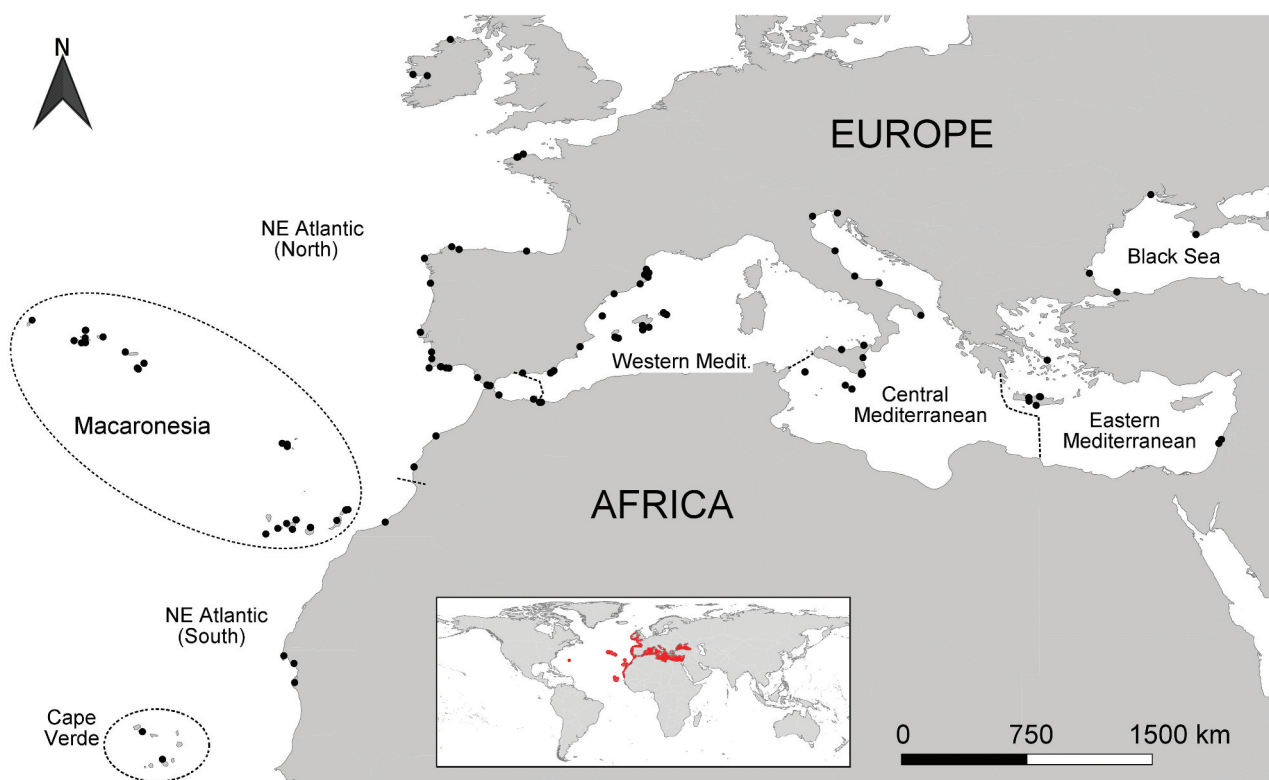

Fig. 1. *Cystoseira* s.l. samples sequenced for the *cox*1 barcoding marker. Dotted lines delimit the major (informal) geographic subdivisions considered in this study within the general North-east Atlantic and the Mediterranean basins. Data obtained from GenBank are also included. The inset depicts the global distribution of *Cystoseira* s.l.

Table 1. Results of Species Delimitation Methods (SDMs). First column lists all MOTUs recovered by jMOTU based on haplogroup discontinuities, and subsequent columns depict the MOTU boundaries as recovered with ABGD, PTP and GMYC SDMs. Lumped MOTUs are highlighted in grey. At the bottom, total number of MOTUs recovered (from left to right *Cystoseira* s.s., *Ericaria* and *Gongolaria*).

jMOTU	ABGD		PTP		GMYC			
	JC69	K80	ML	Bayes	SC	SY	RC	RY
C. foeniculacea	+	+	+		+	+	+	+
C. compressa s.s.	+	+			+	+	+	+
C. pustulata	+	+	+	+	+	+	+	+
C. humilis s.s.	+	+			+	+	+	+
E. selaginoides A	+	+						
E. selaginoides B			+	+	+	+	+	+
E. selaginoides C	+	+						
E. zosteroides	+	+	+	+	+	+	+	+
E. sedoides	+	+	+	+	+	+	+	+
E. dubia	+	+	+	+	+	+	+	+
E. balearica	+	+	+	+				
E. crinita complex	+	+			+	+	+	+
E. brachycarpa s.s.	+		+	+				
E. corniculata	+	+						
G. sonderi	+	+	+	+				+
G. abies-marina	+	+	+	+	+	+	+	+
G. barbata	+	+	+	+	+	+	+	+
'G. susanensis'								
G. baccata	+	+	+	+	+			+
G. usneoides	+	+	+	+	+			+
Gongolaria sp. 1								
G. rayssiae	+	+	+	+	+			+
Gongolaria sp. 2	+	+	+	+	+	+	+	+
G. elegans s.l.					+			+
G. gibraltarica	+	+						
G. nodicaulis			+	+				
G. montagnei s.l.					+			+
TOTAL (4C|10E|13 G)	4|9|7	4|8|7	2|6|8	2|8|8	4|5|9	4|5|3	4|5|3	4|5|9

JC90: Jukes-Cantor distance; K80: Kimura 1980 distance; PTP ML: Maximum Likelihood partition; PTP Bayes: Most supported partition found by simple heuristic search; GMYC S, R, C, Y: Strict or Relaxed Molecular Clock prior, Constant Growth or Yule tree prior.

(635 + 558 nt, used to produce haplotype networks and identify MOTUs), (2) partial sequences, when one of the fragments, or part of it, could not be amplified/sequenced, and (3) published sequences available from GenBank (Supplementary table S2).

jMOTU recovered 4 *Cystoseira* s.s., 10 *Ericaria* and 13 *Gongolaria* MOTUs (Table 1). ABDG analyses recovered 4 *Cystoseira* s.s., 8–9 *Ericaria* but as few as 7 *Gongolaria* spp. PTP and GMYC analyses recovered 2/4 *Cystoseira* s.s., 6–8/5 *Ericaria* and 8/9 *Gongolaria* spp. Altogether, *E. selaginoides* A-C, *G. rayssiae* and *Gongolaria* sp. 1, *G. barbata* and Marzameni's *G. susanensis*, and several tophulose *Gongolaria* (e.g. *G. nodicaulis*, *G. montagnei*) were consistently recovered as conspecific (Table 1). GMYC analyses also recovered *E. crinita* complex and related MOTUs as a single entity, irrespective of the priors selected. Compared with jMOTU, all other species delimitation methods were very conservative and consistently lumped related taxa otherwise recognizable for their distinct morphologies, diagnostic mutations and distinct geographic ranges. Therefore, we adopted, for subsequent phylogeographic and phylogenetic analyses based on discrete taxonomic units, the MOTUs as delimited with jMOTU.

The *Cystoseira* s.s. alignment comprised 88 original *cox*1 sequences (68 complete, 20 partial; GenBank accessions OK480237-324) plus 18 from GenBank (Supplementary table S2). The *cox*1 network and

jMOTU analyses (using cut-off value of six mutations) revealed four MOTUs belonging to two main lineages (Fig. 2a). *Cystoseira foeniculacea* (Linnaeus) Greville was the most genetically divergent and exhibited the highest morphological plasticity throughout its wide Atlantic/Mediterranean distribution (Fig. 2b). Intra-specific diversity, however, was lower than in other congeners and did not correlate with morphological formae (*latiramosa*, *tenuiramosa*, data not shown). A previously undescribed free-living form from Ria Formosa lagoon (southern Portugal), originally presumed to be *Gongolaria barbata* f. *repens* (A.D.Zinova & Kalugina) Sadogurska, was also found to be this species (Supplementary Fig. S2a). The remaining three MOTUs were much more closely related (Fig. 2a). Polymorphic *C. compressa* was sampled from Israel to southern Portugal as well as in the Canary Islands (Fig. 2c). *Cystoseira humilis* Schousboe ex Kützing was sampled in intertidal rock-pools throughout the Atlantic, including Madeira and Canary Islands (Fig. 2d). The third MOTU included samples from Azores, Canary Islands and the Mediterranean traditionally identified as *C. compressa*, *C. compressa* subsp. *pustulata* (Ercegovic) Verlaque, or *C. humilis*, but clearly genetically distinct (Fig. 2a, d).

The *Ericaria* alignment comprised 99 *cox*1 sequences (81 complete, 18 partial, GenBank accessions OK480325-423) plus 20 from GenBank (Supplementary table S2). The *cox*1 network and

Table 2. Intra-MOTU diversity among entities with better phylogeographic representation.

MOTU	Complete sequences	N_{hap}	H_{hap}	π ($\times 10^{-5}$)
Cystoseira foeniculacea	9	3	0.722	116
Cystoseira compressa s.s.	19	6	0.789	265
Cystoseira pustulata	25	7	0.807	212
Cystoseira humilis s.s.	16	4	0.650	134
Ericaria selaginoides compl.	55	16	0.861	371
haplogroup A	40	10	0.756	169
haplogroup B	9	4	0.806	214
Ericaria crinita compl.	10	4	0.778	207
Gongolaria abies-marina	11	5	0.618	88
Gongolaria baccata	9	2	0.500	42
Gongolaria usneoides	8	2	0.536	45
Gongolaria nodicaulis	6	2	0.533	45
Gongolaria montagnei s.l.	11	4	0.491	61

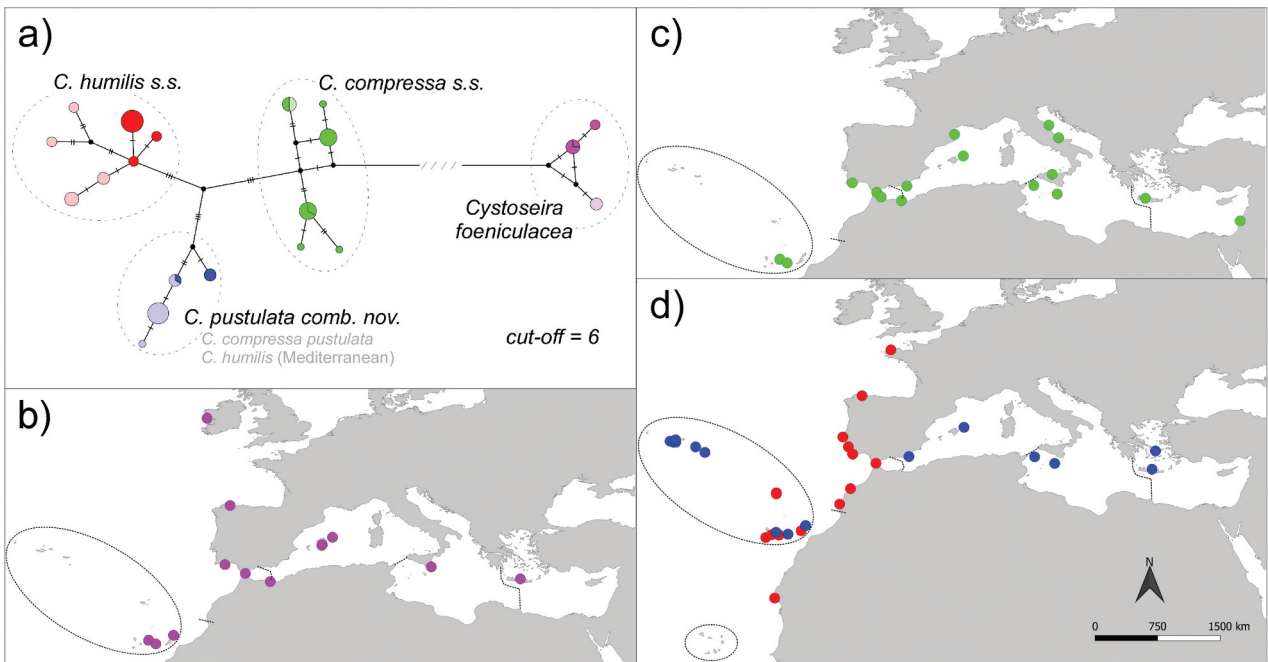N_{hap}: Number of haplotypes; H_{hap}: haplotypic diversity; π: nucleotide diversity.

Fig. 2. Genetic entities and distribution of *Cystoseira* s.s. (**a**) *Cox*1 TCS haplotype network, with dashed circles delimiting inferred MOTUs (see discussion for taxonomic names). Haplotypes are represented by circles sized to their frequency. Pale colours indicate variation endemic to temperate Macaronesia (Azores, Madeira, Canary Islands), strong colours indicate haplotypes sampled elsewhere. Small dashes along the lines connecting haplotypes represent one bp mutation, larger dashes delimit major lineages, and black dots represent internal nodes. (**b–d**) Geographic sampling of each MOTU, using the same general colour code as in (**a**). Dashed lines separate major oceanographic regions as depicted in Fig. 1.

jMOTU analyses (using cut-off value of five mutations) revealed that this genus, of primarily Mediterranean distribution, is composed of, at least, 10 MOTUs belonging to five main lineages (Fig. 3a). *Ericaria zosteroides* (C.Agardh) Molinari & Guiry (sampled in Catalonia, Spain), *Cystoseira sedoides* (Kützing) Piccone (sampled in Pantelleria Island, Sicily) and *C. dubia* Valiante (sampled in Crete, Greece) represented the single extant representatives of their respective lineages. A more diversified Mediterranean lineage included samples originally identified as *E. brachycarpa* (J.Agardh) Molinari & Guiry, *E. crinita* (Duby) Molinari & Guiry, *E. barbatula* (Kützing) Molinari & Guiry, *E. giacconei* D.Serio & G.Furnari (= *Cystoseira hyblaea* Giaccone, from Sicily) and *Cystoseira*

corniculata (Turner) Zanardini (Crete) (Fig. 3a). Samples of *E. brachycarpa* corresponded to two cryptic but genetically well-differentiated entities. One, traditionally identified as *E. brachycarpa* var. *balearica* (Sauvageau) Giaccone (Gómez-Garreta *et al.*, 2001; Mariani *et al.*, 2019), was genetically confirmed from throughout the Balearic Sea and the Sicilian island of Pantelleria, whereas the other was sampled in the northern coast of Sicily and in Crete (Fig. 3c). The latter showed considerable morphological differences (Supplementary fig. S3). Only one of the two *cox*1 fragments was successfully amplified in Sicilian samples, so the actual differences between Sicilian and Cretan populations may be underestimated. Conversely, a single MOTU aggregated samples of *E. crinita* (including the Black Sea's *E. crinita*
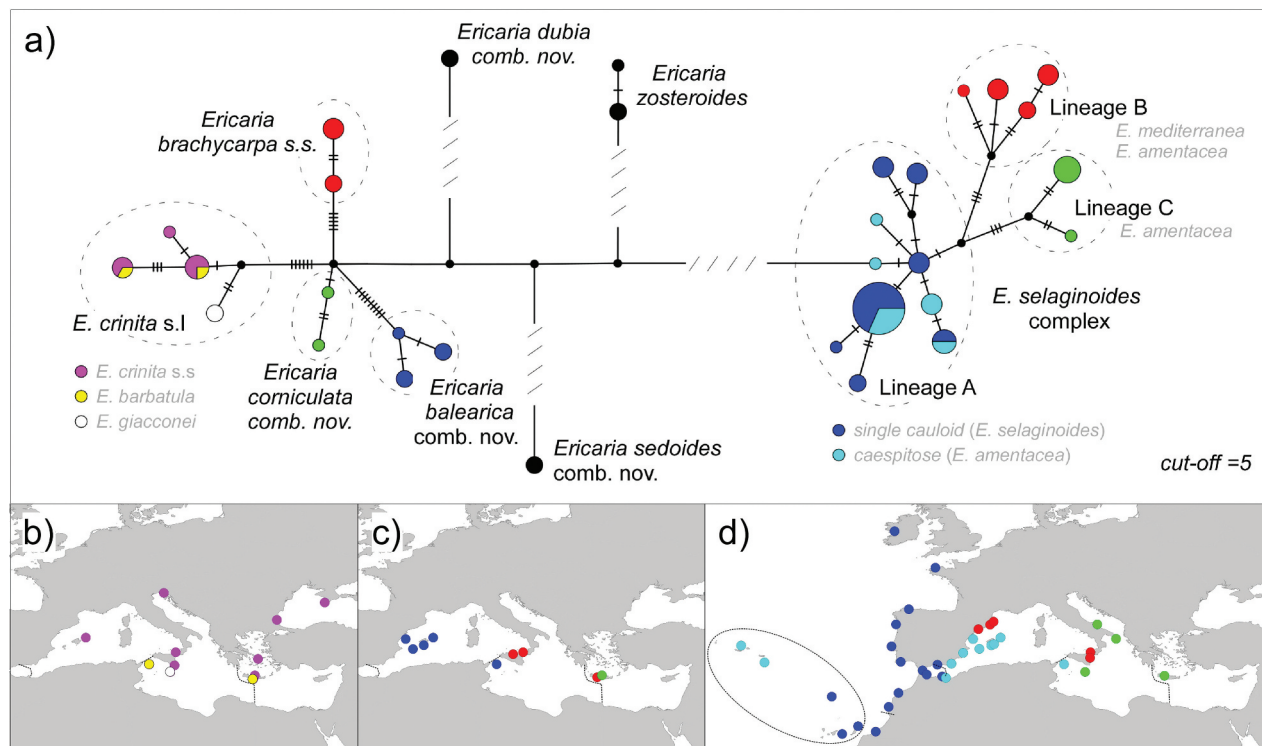
Fig. 3. Genetic entities and distribution of *Ericaria*. (**a**) *Cox*1 TCS haplotype network, with dashed circles delimiting inferred MOTUs (see discussion for taxonomic names). Haplotypes are represented by circles sized to their frequency. Small dashes along the lines connecting haplotypes represent one bp mutation, larger dashes delimit major lineages, and black dots represent internal nodes. (**b–d**) Geographic sampling of each MOTU, using the same colour code as in (a) with (**b**) depicting *E. crinita* s.l., (**c**) *E. corniculata, E. balearica* and *E. brachycarpa* s.s., and (**d**) *E. selaginoides* complex. Dashed lines separate major oceanographic regions as depicted in Fig. 1. *E. dubia, E. zosteroides* and *E. sedoides* were not mapped.

f. bosphorica (Sauvageau) Sadogurska, Neiva & Israel, recognized by some authors as a separate species *E. bosphorica* (Sauvageau) D.Serio & G.Furnari), *E. barbatula* and *E. giacconei* (Fig. 3b). Despite some polymorphism, haplotypes were not correlated with previously described morphological species. For instance, samples of *E. crinita* and *E. barbatula* from Crete showed the same unique regional haplotype despite obvious differences in the apexes of cauloids (Supplementary fig. S4), whereas *E. crinita* from Menorca (Balearic Islands), *E. barbatula* from Pantelleria and *E. crinita*f. *bosphorica* from the Black Sea also possessed identical *cox*1 sequences. Likewise, haplotypes of *E. giacconei* from Sicily were also shared with putative *E. crinita* from the Gulf of Trieste in the Adriatic Sea (MT978054, Sadogurska *et al.*, 2021). Finally, samples originally identified as *E. selaginoides* (Linnaeus) Molinari & Guiry (= *Cystoseira/Carpodesmia tamariscifolia*), *E. amentacea* (C.Agardh) Molinari & Guiry and *E. mediterranea* (Sauvageau) Molinari & Guiry formed, as somewhat expected, three main clusters (herein named haplogroups A, B and C of *E. selaginoides* complex), but with very poor correspondence with a priori morphological identifications. Instead, haplogroup A included single-cauloid Atlantic samples consensually identified as *E. selaginoides*, as well as

caespitose Mediterranean algae from the southeastern Iberian Peninsula, Balearic Islands and Pantelleria identified as *E. amentacea* (Fig. 3d). Haplogroup B grouped samples of *E. mediterranea* from Spanish Catalonia, and caespitose *E. amentacea* from Sicily. Haplogroup C grouped eastern caespitose algae from Malta, the Adriatic and Crete, also identified as *E. amentacea*.

The *Gongolaria* alignment comprised 101 *cox*1 sequences (82 complete, 19 partial, GenBank accessions OK480424-524) plus 24 from GenBank (Supplementary table S2). The *cox*1 network and jMOTU analyses (using cut-off value of three mutations) revealed 13 + MOTUs distributed unevenly in two main clades, herein *Gongolaria* A and B (Fig. 4a). The first comprised two well-defined caespitose species with non-overlapping distributions in the temperate Macaronesian (*G. abies-marina* (S.G.Gmelin) Küntze) and the Cape Verde (*Cystoseira sonderi* (Kützing) Piccone) archipelagos (Fig. 4b). *Gongolaria* B was composed by three main lineages vastly distributed in the Atlantic and the Mediterranean, but very poorly represented in Macaronesia. One comprised two large Atlantic species, *G. baccata* (S.G.Gmelin) Molinari & Guiry and *G. usneoides* (Linnaeus) Molinari & Guiry (Fig. 4c). Another lineage, composed by *G. barbata* (Stackhouse) Kuntze and Sicilian samples previously
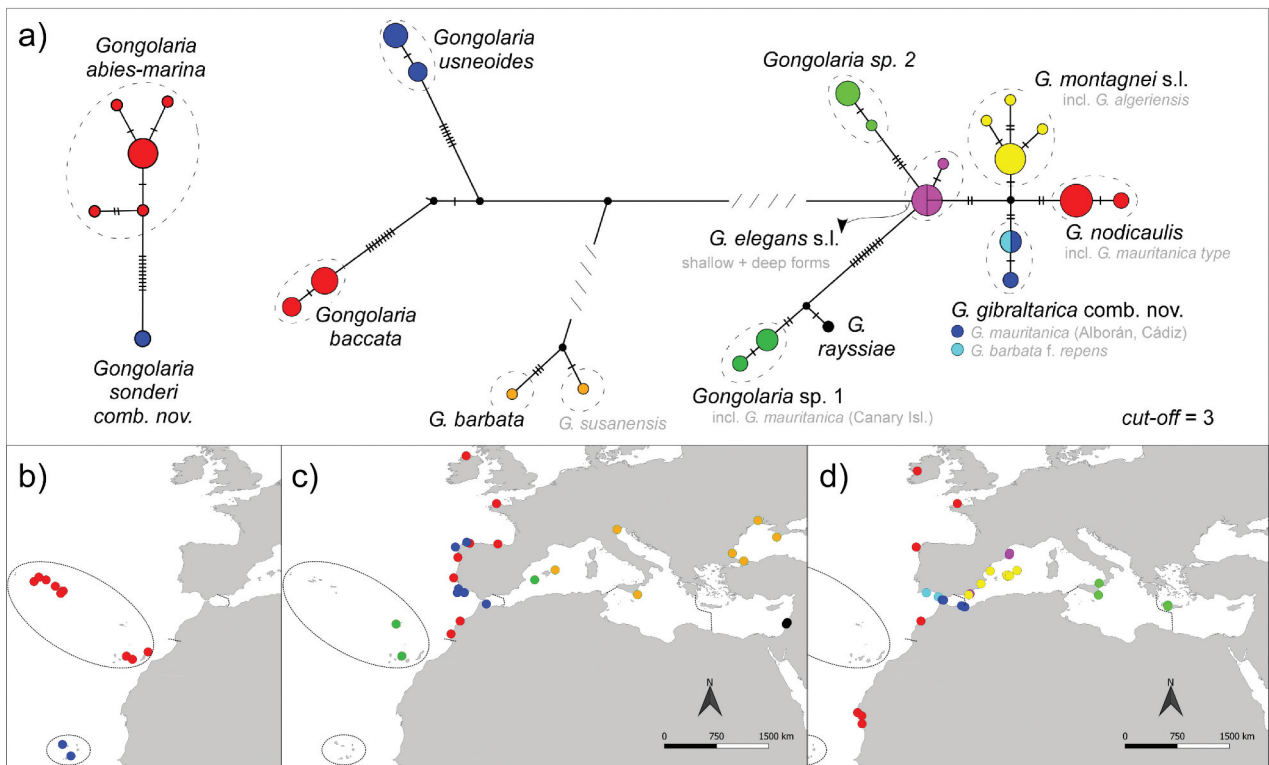
Fig. 4. Genetic entities and distribution of *Gongolaria*. (**a**) *Cox*1 TCS haplotype networks of clade A (left) and clade B (right), with dashed circles delimiting inferred MOTUs (see discussion for taxonomic names). Haplotypes are represented by circles sized to their frequency. Small dashes along the lines connecting haplotypes represent one bp mutation, larger dashes delimit major lineages, and black dots represent internal nodes. (**b–d**) Geographical sampling of each MOTU, using the same colour code as in (a) with (**b**) depicting *G. abies-marina* and *G. sonderi*, (**c**) *G. baccata*, *G. usneoides*, *G. barbata* (incl. Marzameni's '*G. susanensis*'), *G. rayssiae* and *Gongolaria* sp. 1, and (**d**) *G. nodicaulis*, *G. montagnei* s.l., *G. elegans* s.l. (incl. Columbrete's *G. sauvageauana*), *G. gibraltarica* and *Gongolaria* sp. 2. Dashed lines separate major oceanographic regions as depicted in Fig. 1.

identified as *G. susanensis* (Nizamuddin) Molinari & Guiry (Draisma *et al*., 2010), was genetically confirmed throughout the Mediterranean and the Black Sea (Fig. 4c). Finally, the third lineage comprised multiple species complexes with very low genetic differentiation (Fig. 4a), but some phylogeographic signal (Fig. 4c, d). One MOTU, comprising tophulose Atlantic algae traditionally identified as *G. nodicaulis* (Withering) Molinari & Guiry (*C. granulata* auctorum in older literature), was sampled from Ireland to Mauritania (Fig. 4d). Another MOTU, comprising algae traditionally identified as *G. mauritanica* (Sauvageau) Molinari & Guiry (Gómez-Garreta *et al*., 2001; Bermejo *et al*., 2015), was sampled around Tarifa (Spain) and Nador (Morocco) (Fig. 4d). Free-living forms from Ria Formosa, Cádiz Bay and Nador lagoons, previously identified as *G. barbata* f. *repens* (Hernández *et al*., 2010; Ramdani *et al*., 2015, Supplementary fig. S2b, c) were also found to be within this entity (Fig. 4a, d). Two additional MOTUs were sampled along the Mediterranean coasts of Spain. The first, *G. montagnei* (J.Agardh) Küntze, comprised very polymorphic samples originally identified as *Cystoseira spinosa* (= *Treptacantha montagnei*, *T. ballesterosii*) and *C. algeriensis* (= *Gongolaria algeriensis* (Feldmann)

Molinari & Guiry) (Sales & Ballesteros, 2009; Sousa *et al*., 2019a; Jódar-Pérez *et al*., 2020), from both very shallow to deeper (−20 m) environments (Fig. 4a, d). The other comprised morphologically distinct collections of shallow and deeper-water *G. elegans* (Sauvageau) Molinari & Guiry (Supplementary fig. S5; Mariani *et al*., 2019; Medrano *et al*., 2020), as well as an overwintering collection of *G. sauvageauana* (Hamel) Molinari & Guiry from Columbretes (Valencia) (Fig. 4a, d). Another related MOTU, referred to as *Gongolaria* sp. 2, comprised a relatively homogeneous group of samples from Crete and Sicily (0–4 m deep) with characteristically swollen cauloid apexes (Supplementary fig. S6), but previously identified as *G. elegans* (Draisma *et al*., 2010), or locally (Crete) as *Cystoseira spinosa* (= *G. montagnei*) (Fig. 4a, d). Finally, the most divergent complex included samples of the Levantine-endemic *G. rayssiae* (Ramon) Molinari & Guiry (Mulas *et al*., 2020) and from a closely related entity from Macaronesia (Fig. 4a, c). This latter, referred to as *Gongolaria* sp. 1, comprised algae from Tenerife (Canary Islands) originally identified as *G. mauritanica*, and *Gongolaria* sp. from Madeira. A 12 m deep overwintering *Gongolaria* from the Balearic Sea, tentatively identified as *G. montagnei*,

also possessed this haplotype. Despite the single base-pair difference, these two rare intertidal entities were morphologically very distinct (Supplementary fig. S7).

Several voucher specimens, encompassing the range of observed morphological variation of each MOTU, were deposited in the herbarium of the University of Algarve (ALGU-ALGAS), under vouchers listed in Supplementary table S3.

Intra-specific diversity and barcoding gap

Intra-MOTU diversity was very variable, as shown for 10 selected MOTUs with more than six complete sequences and wide geographic coverage (Table 2). *Cystoseira* s.s. spp. revealed appreciable haplotypic and nucleotide diversity ($100 < \pi \times 10^5 < 280$) and phylogeographic structure, with most MOTUs exhibiting unique variation in the temperate Macaronesian archipelagos (Fig. 2a). Among *Ericaria*, *E. crinita* complex was quite variable ($\pi \times 10^5 = 207$), but apparently lacking significant phylogeographic structure or correspondence between MOTUs and morpho-species. By all measures, the Atlantic/Mediterranean *E. selaginoides* complex was, by far, the most variable ($\pi \times 10^5 = 371$) and geographically structured taxa. In haplogroup A, the most comprehensively sampled, one haplotype dominated in the Atlantic and western Mediterranean, but diversity was still high ($\pi \times 10^5 = 169$), with the most differentiated samples being those of Santa Maria Island (Azores). *Gongolaria* spp. showed the lowest polymorphism ($40 < \pi \times 10^5 < 90$), with 1–2 haplotypes typically dominating MOTU's entire ranges.

Reflecting variable polymorphism, maximum intra-MOTU sequence divergences ($K2P \times 10^2$) were higher among *Cystoseira* s.s. (0.590) and *Ericaria* (0.506) than among *Gongolaria* MOTUs (0.337). Intra- and inter-MOTU distances, however, overlapped marginally in all three genera (Fig. 5a–c), owing to the low divergence between many MOTU-pairs within lineages. Despite low support for a barcoding gap, maximum intra-MOTU distances were, as a rule, lower than minimum inter-MOTU distances (Fig. 6). Exceptions included the *E. selaginoides* complex, where fewer haplotypes of polymorphic haplogroup A were as divergent or more divergent than they were to haplotypes belonging to haplogroup B. *Gongolaria montagnei* complex and *G. nodicaulis* were another exception where intra- vs inter-MOTU distances overlapped. Despite shallow *cox*1 divergence, most mutations were taxon-specific and thus diagnostic.

Organelle phylogeny

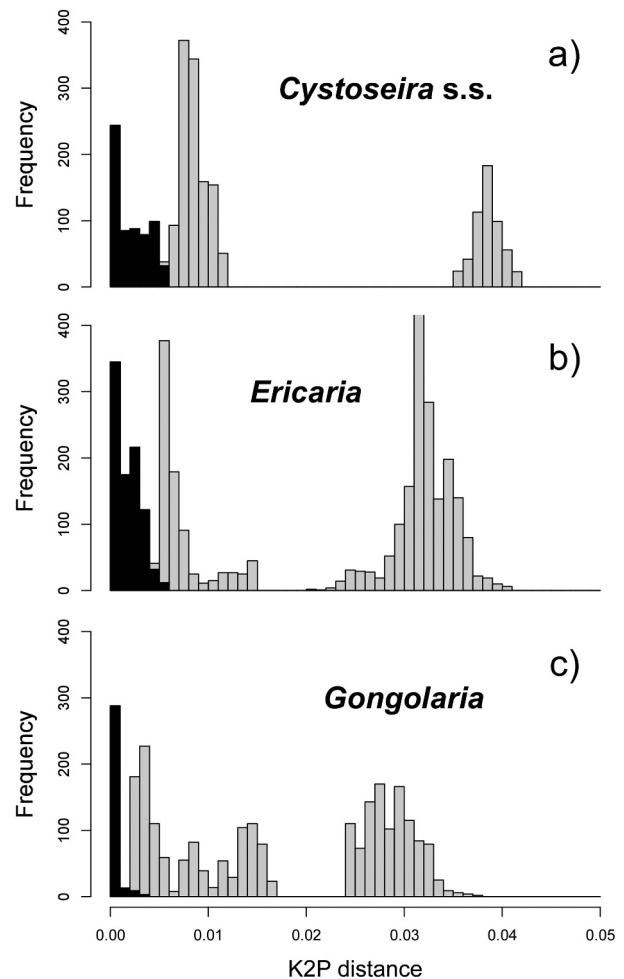Final *cox*1 (outgroups GenBank OK480525-27), *cox*3 (GenBank OK545756-91), *nad*1 (GenBank OK545828-

Fig. 5. Intra-generic divergence and barcoding gaps in *Cystoseira* s.l. From top to bottom absolute frequencies in (**a**) *Cystoseira* s.s., (**b**) *Ericaria* and (**c**) *Gongolaria*, with intra- (black) and inter-MOTU (grey) pairwise K2P distances. Note the absence of a clear barcoding gap in all three genera.

63) and *psa*A (GenBank OK545792-827) alignments were 1193 (635 + 558), 538, 764 and 810 bp long, respectively. Polymorphism was relatively high and even among the mtDNA markers analysed, but much lower in the cpDNA marker which offered very limited resolution within shallow MOTUs complexes. For instance, *E. selaginoides* haplogroup A (sampled as *E. amentacea*) and *E. crinita* from Menorca differed by 18, 19, 16 and 20 mutations in *cox*1-I, *cox*1-II, *cox*3 and *nad*1, but only by 10 in *psa*A fragment, whereas the former and *E. selaginoides* haplogroup C (also sampled as *E. amentacea*) differed by 3, 4, 3, 3 vs 1 mutations for the same markers. Organellar genomes are maternally transmitted as linked gene-blocks; as expected, individual mtDNA gene trees (but also *psa*A) showed similar topologies in exploratory analyses (Supplementary fig. S8–S10), hence only the multi-gene trees reconstructed from concatenated alignments (3305 nt supermatrices) are discussed in detail (Fig. 7). Bayesian and ML trees showed similar topologies and nodes support. Among *Cystoseira* s.s., the phylogenetic tree fully supported the
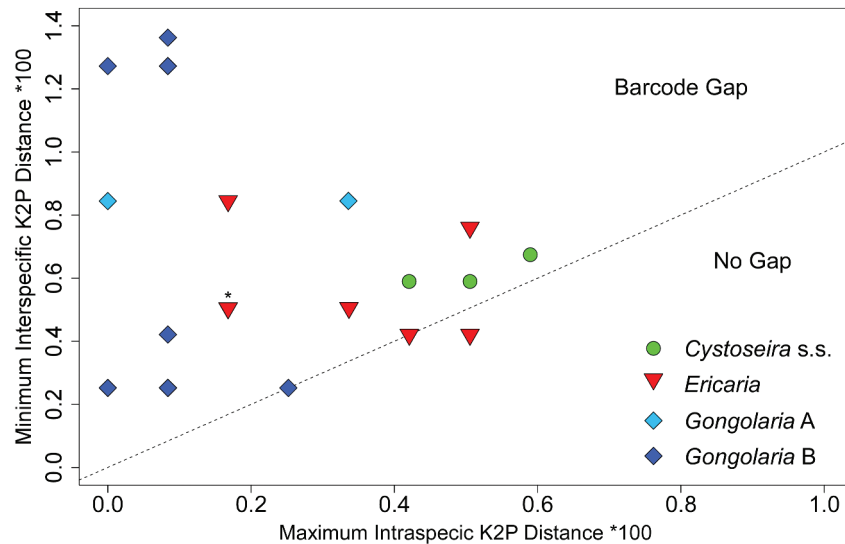
Fig. 6. Barcoding gaps in *Cystoseira* s.l. Maximum intra-MOTU vs minimum inter-MOTU K2P distances among *Cystoseira* s.l. Asterisk (*) marks superimposed data.

monophyly of *C. humilis*, *Cystoseira* sp. (morphologically identified as *C. compressa* subsp. *pustulata*) and *C. compressa* (PP/BS = 1.00/100), but nodal support for a closer relationship of the first two was very weak (PP/BS = 0.53/64) (Fig. 7a). Relationships between the five major lineages of *Ericaria*, previously inferred from *cox*1 networks, also remained unresolved. Basal nodes containing *C. dubia* and *E. zosteroides* were poorly supported (PP < 0.60, BS < 45) and *C. sedoides*, *E. selaginoides* complex and *E. crinita*/*E. brachycarpa* lineage also formed a poorly supported polytomy (Fig. 7b). Within the *E. selaginoides* complex, support for a closer relationship of Mediterranean haplogroups B and C was high (PP/BS = 0.99/92). The *E. crinita* complex, *C. corniculata* and *E. brachycarpa* s.s. formed a fully supported node (PP/BS = 1.00/91), but support for a closer relationship of the latter two was much weaker (PP/BS = 0.59/67). As expected, the phylogenetic tree of *Gongolaria* fully resolved the two divergent clades A and B (Fig. 7c). Within the latter, concatenated data retrieved *G. barbata* s.l. as the most basal lineage, but with very poor nodal support (PP/BS = 0.57/62). The node containing *G. nodicaulis* and other closely related MOTUS was well supported (PP/BS = 1.00/98), forming with western Mediterranean *G. elegans* a wider group (PP/BS = 1.00/98) sister to the eastern *Gongolaria* sp. 2.

Discussion

The use of a longer *cox*1 barcode and the analyses of the most geographically and taxonomically comprehensive panel of samples of *Cystoseira* s.l. to date, allowed unprecedented resolution to identify (and in some cases tentatively map) major genetic entities. In particular, higher haplogroup discontinuities and number of diagnostic mutations and importantly

phylogeographic signal contributed to significantly improve phylogenetic resolution within shallow species complexes when compared with previous studies using shorter *cox*1 fragments and other markers with lower resolution (23S, mt23S-tRNA Val spacer, psbA; Rožić *et al.*, 2012; Orellana *et al.*, 2019; Sousa *et al.*, 2019a; Jódar-Pérez *et al.*, 2020; Mulas *et al.*, 2020; Sadogurska *et al.*, 2021). In addition, the analyses of a diversified collection of samples for the same barcode marker clearly facilitated the detection of putative cases of misclassification, cryptic taxa and excess splitting, particularly within the *Ericaria brachycarpa*/*crinita* and the tophulose *Gongolaria* lineages, and among free-living lagoon forms. Underlining the utility of the approach, at least 27 MOTUs were recovered based on haplogroup discontinuity. Many matched, for the most part, currently recognized taxa (as delimited by morphology and geography), including a few unassigned *Cystoseira* s.l. spp. (e.g. *Cystoseira sedoides*, *C. dubia*), which, for the first time, are assigned to their correct genus. Some, on the other hand, conflicted with classic species circumscriptions and required more substantial taxonomic changes, such as the re-definition, reinstatement and even recognition of new taxa, with interesting taxonomic, biogeographic and evolutionary implications.

Updates to *Cystoseira* s.s. diversity, biogeography and taxonomy

Cystoseira sensu stricto was the least speciose genus and the one comprising fewer lineages (only 2). All four recognized entities were considerably polymorphic and exhibited wide distributions in the Atlantic, Mediterranean or both. *Cystoseira foeniculacea*, the sole representative of its lineage, illustrated particularly well the mismatch between morphological plasticity
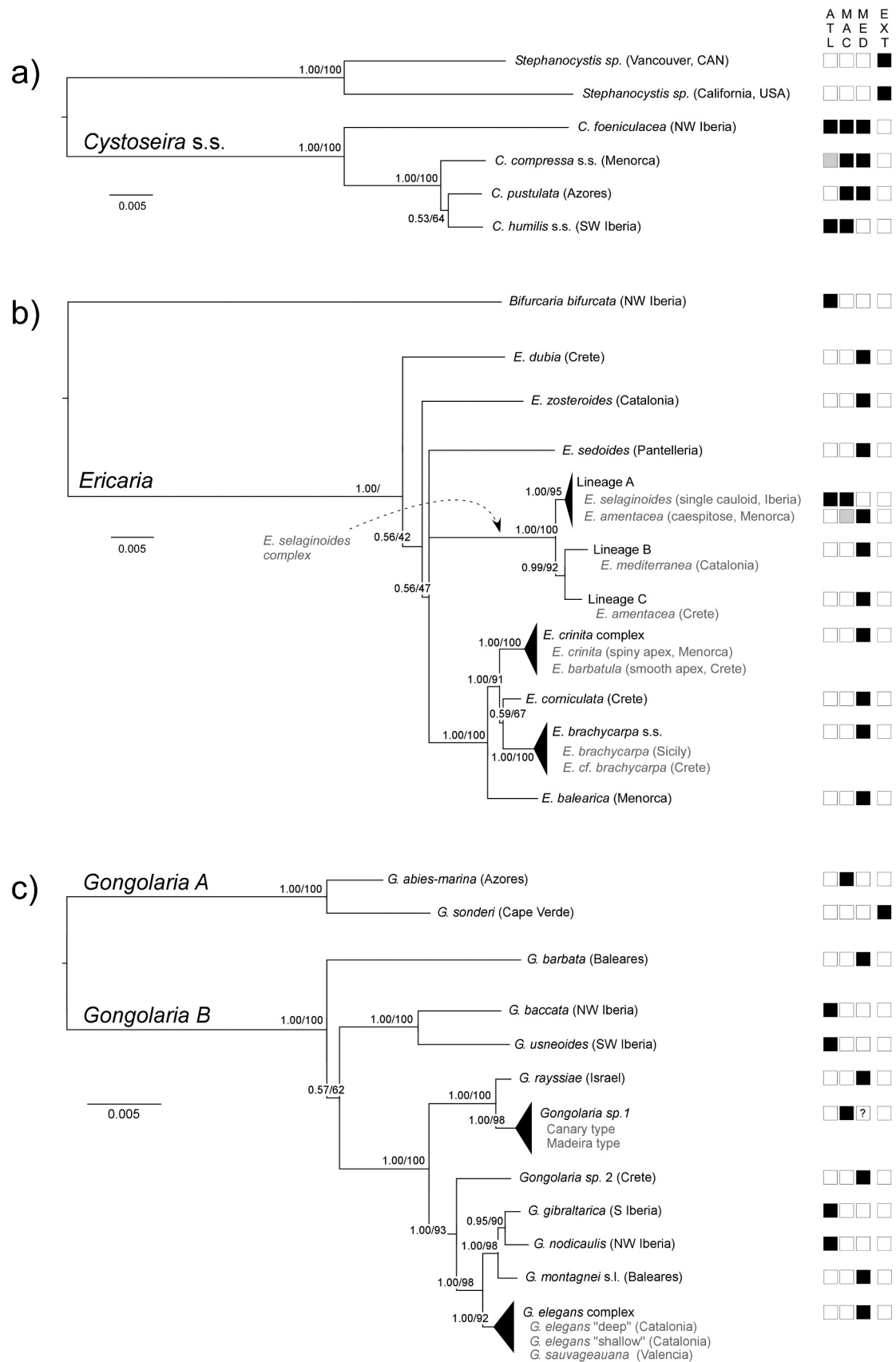
Fig. 7. Organelle phylogenies. Bayesian 50% majority-rule consensus tree of (**a**) *Cystoseira* s.s., (**b**) *Ericaria* and (**c**) *Gongolaria* reconstructed with four concatenated plastid genes (*cox*1, *cox*3, *nad*1 and *psa*A). Numbers near the nodes are Bayesian posterior probabilities (left) and maximum likelihood bootstrap support values (right). Horizontal triangles represent collapsed branches, with length (horizontal) representing the distance from the branches' common node to the tip of the longest branch, and height (vertical) scaled to the number of (unique) sequences collapsed. The panel on the right summarizes the general genetically confirmed distribution of each MOTU. ATL: Atlantic (continental); MAC: Temperate Macaronesia Archipelagos; MED: Mediterranean; EXT: Elsewhere. (black: present; white: not detected; grey: present only marginally; ?: data insufficient).
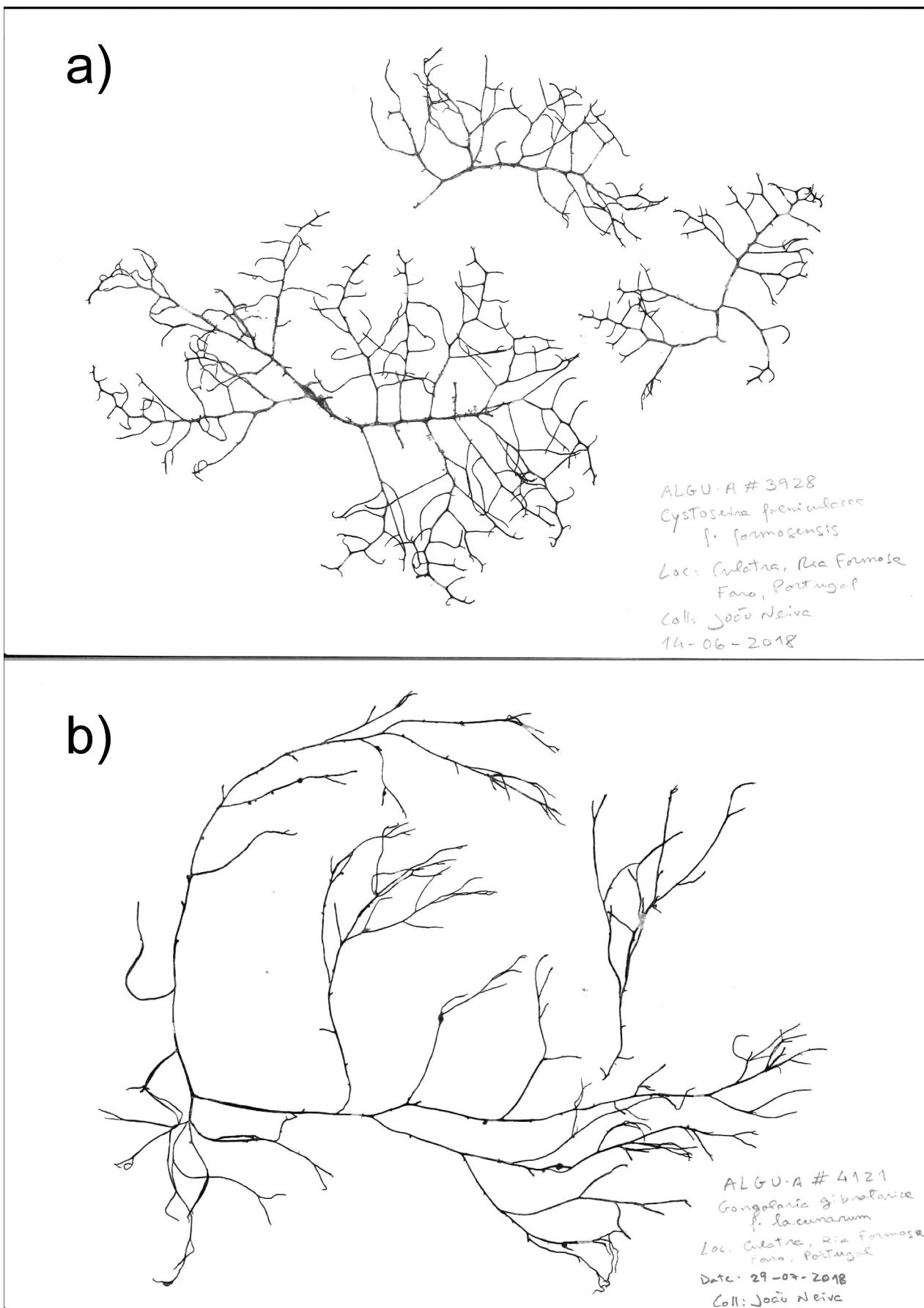
Fig. 8. New free-living *Cystoseira* taxa. (**a**) Holotype of *Cystoseira foeniculacea* f. *formosensis*; (**b**) Holotype of *Gongolaria gibraltarica* f. *lacunarum.*

and genetic polymorphism found in many species. Lack of genetic differentiation, and co-existence of disparate morphological varieties (e.g. typical morphotypes with *C. foeniculacea* f. *latiramosa*), strongly suggests that the striking plasticity of this species is mainly environmental, a hypothesis that should be further investigated using other classes of markers. Interestingly, several lagoon populations (Menorca, Nador), corresponding to *C. foeniculacea* f. *tenuiramosa*, were found epiphytizing the seagrass *Cymodocea nodosa* (Ucria) Asch. The newly discovered free-living form from the Ria Formosa lagoon (southern Portugal), here named *C. foeniculacea* f. *formosensis* Neiva & Serrão forma nov., exhibited an extremely simplified morphology, lacking anchoring holdfasts, true cauloids and the characteristic spines present in other free-living forms of *C. foeniculacea* (e.g. *C. foeniculacea* f. *dubia* (Ercegovic) Bouafif, Verlaque & Langar (Bouafif *et al.*, 2016)). This forma co-occurred with another free-living species (assigned to *Gongolaria gibraltarica*, see below) from which it could be easily distinguished by its markedly compressed divaricate branches that fragmented easily. It is presumed to propagate only clonally, as no reproductive structures were ever observed. Algae from Galicia and Ireland lacked the dense cover of spines along cauloids so characteristic of the species, and thus could be recognized as a separate variety as well.

In the second lineage, barcoding data clearly supported a third MOTU in addition to *C. humilis* and *C. compressa*. In the Mediterranean Sea, this distinct entity has traditionally been regarded as a subspecies of *C. compressa* (*C. compressa* subsp. *pustulata*; Sales & Ballesteros, 2009; Thibaut *et al.*, 2015; Bouafif *et al.*, 2016) or included within *C. humilis* (Cormaci *et al.*, 1992, 2012; Gómez-Garreta *et al.*, 2001; Draisma *et al.*, 2010; Jódar-Pérez *et al.*, 2020). Both Sousa *et al.* (2019*a*) and Orellana *et al.* (2019) have previously recognized it as a separate entity in face of obvious sequence differences, the former proposing the name *C. humilis* var. *humilis* (a simple autonym of *C. humilis*, and thus invalid) and the latter reinstating the name *C. aurantia* Kützing. *Cystoseira aurantia* was very scantily described from the Gulf of Trieste (Adriatic Sea) and is generally regarded as the free-living ecotype *Gongolaria barbata* f. *repens* (= *Cystoseira barbata* f. *aurantia* (Kützing) Giaccone). The very distinct morphology and ecology of the Canary taxon (attached algae inhabiting intertidal rock-pools) and complete absence of genetic evidence suggesting conspecificity with the Adriatic taxon makes the nomenclatural choice rather enigmatic. Considering all available data (genetic, morphological, biogeographic), it is more appropriate to elevate *C. compressa* subs. *pustulata* (basionym *C. abrotanifolia* subsp. *pustulata*) to species level as *C. pustulata* (Ercegovic) Neiva & Serrão, comb. nov. Recognizing this species requires a narrower circumscription of *C. compressa* (*C. compressa* s.s., hereafter excluded from the Azores and excluding subsp.

pustulata) and *C. humilis* (*C. humilis* s.s., hereafter seemingly excluded from the Mediterranean). Abundant and prominent cryptostomata and a pseudo-caespitose habit, that according to Cormaci *et al.* (2012) characterize *C. humilis* v. *humilis*, seem the best features to discriminate *C. pustulata* from closely related species, but it is still unclear if these characters are constant throughout the vast range of these species. In the Mediterranean, *C. pustulata* often occurs in sympatry with *C. compressa*, although normally restricted to more sheltered (or deeper) positions. In the Azores this was, in conjunction with *G. abies-marina*, the dominant species, but was restricted to intertidal rock-pools (DMF, pers. obs.). *Cystoseira humilis* was found in high-intertidal tide pools and, where coexisting (southern Iberia, Canary Islands), was always found higher on the shore than *C. compressa*. Interestingly, all four *Cystoseira* s.s. species co-occurred in the Canary Islands. Temperate Macaronesia clearly represents a diversity hotspot for the genus, harbouring unique genetic variation in all four species (Fig. 2a) that should be further investigated.

Updates to *Ericaria* diversity, biogeography and taxonomy

Ericaria is, as noted earlier, primarily a Mediterranean genus, with a single MOTU (*E. selaginoides* haplogroup A) occurring throughout the North-east Atlantic and Macaronesia (Fig. 3d). This genus exhibited considerable diversity, including five lineages and at least 10 MOTUs. Newly available molecular data allowed transferring a few more species to this genus, namely *Cystoseira sedoides*, hereafter *Ericaria sedoides* (Desfontaines) Neiva & Serrão, comb. nov.; *C. dubia*, hereafter *Ericaria dubia* (Valiante) Neiva & Serrão, comb. nov.; *C. corniculata*, hereafter *Ericaria corniculata* (Turner) Neiva & Serrão, comb. nov.; and also supported the recent transfer of *E. giacconei* to *Ericaria* (Serio & Furnari, 2021). The first two, together with deep-water *E. zosteroides*, represented the single extant representatives of their respective Mediterranean lineages, and all three showed either geographic endemism or specialized habitats. Their phylogenetic value, coupled with perceived vulnerability, confers upon them special conservation importance. These species were very poorly sampled for barcoding, but all three have very distinctive morphologies and low risk of confusion with other species, so that reported distributions are probably more accurate than for other *Ericaria*.

Barcoding data also requires recognizing *C. brachycarpa* var. *balearica* as a cryptic species distinct from *E. brachycarpa*. Both entities, hereafter *Ericaria balearica* (Sauvageau) Neiva, Ballesteros & Serrão 2021, comb. nov. (type locality: Las Isletas, Mallorca), and *E. brachycarpa* s.s. (type locality: Salerno, Campania, Italy) were present around Sicily

(incl. Pantelleria), but because of limited sampling, their eastward and westward range limits can only be guessed. Future studies should refine their distributions and potentially identify areas of range overlap and identify constant diagnostic morphological differences between the two. A closer look at *E. brachycarpa* s.s. is particularly warranted. This entity was only genetically confirmed from the northern coast of Sicily and southern Crete, where local populations showed considerable morphological differences. Cretan algae showed more robust cauloids and spiny apexes and primary branches and were initially hypothesized to correspond to another taxon, *Cystoseira crinitophylla* Ercegović. One of the *cox*1 fragments was impossible to amplify in the Sicilian populations, so that the actual degree of differentiation between the two could potentially have been underestimated. The hypothesis of two distinct entities within *C. brachycarpa* s.s. cannot be discarded, particularly considering morphological differences and incomplete *cox*1 data, but negligible *cox*3 and *nad*1 differentiation seem to support instead a single polymorphic taxon whose differences may reflect instead their distinct shallow and exposed (< 0.5 m; Sicily) vs deeper (≥ 4 m; Crete) environments.

Barcoding/phylogeographic data were also incompatible with current circumscriptions of *E. crinita* and *E. barbatula*. Sadogurska *et al.* (2021) previously showed that *cox*1 haplotypes within this complex were not correlated with the main feature used to distinguish the two morphospecies: the presence of spinose (*E. crinita*) vs smooth (*E. barbatula*) cauloid apexes, and put forward the hypothesis of these taxa being conspecific. Serio & Furnari (2021) disputed such interpretation taking into account the important morphological differences between those species and the absence of sound molecular data. The argument applied also to *E. crinita* f. *bosphorica* (= *E. bosphorica*), a taxon seemingly endemic to the Black Sea that can grow a metre high, has a smooth apex (unlike typical *E. crinita*), and displays, uniquely within this MOTU, numerous aerocysts. In the same line, *E. crinita* from Crete exhibited an extremely short and compact shrubby habit, apparently due to heavy grazing, but haplotypic differences with respect to other, more typical *E. crinita* were again shared with local *E. barbatula*. Here, a longer fragment of *cox*1, as well as additional *cox*3 and *nad*1 sequence data, confirmed that multiple haplotypes are shared between these taxa, but also between putative *E. crinita* (from the Adriatic) and *E. giacconei*, a species so far reported only from Sicily and Tunisia. From a phylogenetic perspective, these data seem to support a single, very polymorphic entity. A possibility remains, nonetheless, that reproductive isolation is recent (or introgression pervasive) and multiple biological species exist notwithstanding shared/closely related mtDNA haplotypes. Nuclear microsatellites or other co-dominant gene-flow markers are essential to disentangle these hypotheses and should prove particularly informative in settings where multiple entities occur in sympatry or close proximity (Coyer *et al.*, 2011; Tellier *et al.*, 2011*b*; Neiva *et al.*, 2018). If future studies confirm excessive species recognition, *E. barbatula*, *E. bosphorica* and *E. giacconei* should be downgraded to varieties of *E. crinita* s.l., the name with priority (originally described by Duby in 1830).

Additional studies are also required to clarify the number and boundaries of species within the *E. selaginoides* complex. The *cox*1 data retrieved three relatively well defined phylogroups with very little correspondence to currently accepted species – *E. selaginoides*, *E. mediterranea* and *E. amentacea*, at least in their present circumscriptions. Haplogroup A, for instance, spread from the Atlantic to the Balearic Sea and adopted morphologies of both *E. selaginoides* (single cauloid) and *E. amentacea* (caespitose). Likewise, samples of *E. amentacea* belonged, depending on geography, to either haplogroup A (western Mediterranean), B (Sicily) or C (eastern Mediterranean). Another biogeographic-scale study employing microsatellites also found that genetic groups did not match accepted morpho-species. Instead, genetic groups seemed to reflect complex patterns of vicariance and co-ancestry (resulting from glacial-interglacial range-shifts) and more recent gene-flow and secondary contact (Bermejo *et al.*, 2018). The latter authors concluded that the three taxa were better regarded as a single, very polymorphic, species complex. The *cox*1 haplogroups certainly reinforce the limited diagnostic value of habit (caespitose vs non-caespitose) type, but haplogroups A–C also did not show any correspondence with the major microsatellite clusters found in that study. However, unlike the *E. crinita* complex described above, *cox*1 sequences revealed a clear phylogeographic signal, with haplogroups occupying specific geographic ranges and, as far as sampling allowed, little evidence for shared haplotypes and broad overlapping ranges. In a range of North Atlantic fucoids and kelps, similar patterns have been associated with vicariance/secondary contact and other range-shift dynamics associated with glacial/interglacial cycles (Neiva *et al.*, 2016, 2018). The strong phylogeographic structure could provide some basis for giving these haplogroups some type of taxonomic recognition. By virtue of their uniparental inheritance, organelle markers tend to assort faster following vicariance and/or reproductive isolation but are also more prone to introgression and organelle capture associated with gene surfing and/or selective sweeps (Neiva *et al.*, 2010; Nicholls *et al.*, 2012). Considering the pitfalls of a single marker and

the glaring incongruence between morphology, microsatellites and mtDNA, more data are necessary to decipher the nature and best taxonomic treatment for these phylogeographic entities. Refining haplogroups' distributions and particularly investigating background genomic differentiation and gene-flow along phylogeographic contact zones are likely to be particularly useful in this regard.

Updates to *Gongolaria* diversity, biogeography and taxonomy

Gongolaria, with 13+ MOTUs identified, was the most diversified genus. Within clade A, genetic data confirmed close relationship of the Cape Verde's endemic *Cystoseira sonderi* and *G. abies-marina*. The former is here referred to as *Gongolaria sonderi* (Kützing) Neiva, João Soares & Serrão, comb. nov. Within clade B, *G. baccata* and *G. usneoides* represented two well-defined morphological and genetically supported species of Atlantic distribution (the latter also with records in the Mediterranean) with limited scope for confusion. The lineage comprising *G. barbata*, another relatively well-defined morphospecies, and putative *G. susanensis*, was less well-sampled, but barcoding helped narrow down its circumscription. For instance, all free-living forms found at the Nador (Ramdani *et al.*, 2015), Cádiz (Hernández *et al.*, 2010) and Ria Formosa lagoons, originally identified as *G. barbata* f. *repens*, proved to be other species (*Cystoseira foeniculacea*, *Gongolaria gibraltarica*, see further below). These results show that a wider range of species can adopt a free-living habit in low-energy environments and make *G. barbata* an exclusively Mediterranean (including Black and Azov Seas) species. Less clear is the status of putative *G. susanensis*. The original identification of the algae from Marzameni has been disputed (Bouafif *et al.*, 2014), as its morphology (especially the pseudo-caespitose habit) does not fully conform with the original description of *G. susanensis* from Libya (Nizamuddin, 1985). After observation of this population, we are strongly inclined to consider them *G. barbata*. The genetic discontinuity (4 mutations) between Menorca (*G. barbata*) and Marzameni (putative *G. susanensis*) sequences nonetheless supported two MOTUs. However, because of limited sampling (only these two sites with complete cox1 sequences), it is not clear if the discontinuity is real (supporting two species or at least deep phylogeographic structure), or if it simply reflects unsampled intermediate haplotypes (ultimately collapsing into a single polymorphic MOTU). Barcodes of typical, single-cauloid Sicilian *G. barbata* or typical caespitose *G. susanensis* should quickly settle this issue.

The remaining lineage, with seven MOTUs, was by far the most speciose, but species differences were often very shallow. Taking into consideration the low resolution of *cox*1 (when compared with the other two genera), and the perceived incomplete sampling of much of the Mediterranean, recovered diversity is likely to be under-estimated to some extent. In the Atlantic, the *G. nodicaulis* haplogroup was sampled from Ireland to Mauritania. The species was quite common in the Banc D'Arguin, growing on sheltered rocky platforms, but thriving also on soft-bottoms attached to shells, pebbles and frequently on tubes formed by encrusting coralline algae covering decaying *Cymodocea nodosa* rhizomes. *Gongolaria nodicaulis* was very common in shallow waters and beach drift around Nouadhibou, where another species – *G. mauritanica*, was originally described (as *Cystoseira mauritanica* Sauvageau in Hariot (1911), see also Gómez-Garreta & Ribera, 2002)). Considering the original description – 'la végétation du *Cystoseira mauritanica* est comparable à celle des *C. granulata* [*sensu* Greville, = *G. nodicaulis*]', the observed plasticity (e.g. regarding plant and tophule size) of local, North African and European *G. nodicaulis*, and the local and regional ubiquity and abundance of *G. nodicaulis*, we interpret these tophulose taxa as conspecific. Sauvageau described *G. mauritanica* as dioecious, but he only studied two young individuals and a few fragments, and this mating system has never been reported in any other *Cystoseira* s.l. We retain the name *G. nodicaulis*, as *Fucus nodicaulis* Withering has nomenclatural priority.

Algae from around the Gibraltar Strait and the Moroccan coasts of the Alboran Sea, also recognized in the recent literature as *Cystoseira mauritanica* (Gómez-Garreta *et al.*, 2001; Bermejo *et al.*, 2015), represented a related but genetically distinct entity, which is excluded from the synonymy above. Therefore, *Cystoseira gibraltarica* (Sauvageau) P.J.L. Dangeard (basionym: *Cystoseira selaginoides* var. *gibraltarica* Sauvageau; Lectotype locality: Algeciras (Gómez-Garreta & Ribera, 2005)), a name in use in earlier literature (González & Conde, 1993), is hereafter recovered as *Gongolaria gibraltarica* (Sauvageau) Neiva, Bermejo & Serrão, comb. nov. The core distribution of this species extended to southern Portugal as free-living lagoon algae traditionally identified as *G. barbata* f. *repens* (Hernández *et al.*, 2010; Ramdani *et al.*, 2015). These polymorphic unattached formae (e.g. with respect to size, branch widths and abundance of aerocysts) from Ria Formosa, Cádiz and Nador coastal lagoons are here collectively reclassified as *Gongolaria gibraltarica* f. *lacunarum* Neiva & Serrão, forma nov. The geographic limits of this entity are unclear, but it is hypothesized to extend to poorly sampled areas of western Algeria. It may also be the case that other populations of putative *G. barbata* f. *repens* from for example Catalonia

(Mariani *et al.*, 2019) and Tunisia (Bouafif *et al.*, 2016), where the typical attached form of *G. barbata* is absent or rare, are actually this species, which would expand considerably its range into the Mediterranean.

Algae from the Canary Islands, also traditionally recognized as *G. mauritanica* (as *Cystoseira/Treptacantha mauritanica* (Gómez-Garreta *et al.*, 2001; Orellana *et al.*, 2019)), belonged to another divergent MOTU, and thus were also excluded from the synonymy with *G. nodicaulis*. This entity, related to the Levantine-endemic *G. rayssiae*, also included a population from Madeira with a completely different morphology. Both were found in intertidal rock-pools, but the Canary population was much spinier, iridescent, and cauloids were less well developed and apparently lacked tophules. These populations showed negligible differences in all organelle markers sequenced (1–2 mutations). Assuming conspecificity and no introgression (from for example *G. abies-marina*, a co-occurring species with spiny and often iridescent fronds), these differences reveal extreme and disconcerting level of morphological plasticity. Notably, similar plasticity has been documented in related *G. rayssiae* (Mulas *et al.*, 2020). Only perennial structures could be observed in the single wintering Mediterranean population, but its presence at 12 m deep in the Balearic Sea further suggests a broader ecology and cryptic distribution beyond Macaronesia. Unlike *G. gibraltarica*, no earlier name seems to exist for this entity, hereafter referred to as *Gongolaria* sp. 1. Its apparent rarity, even in Macaronesia, and its suspicious level of morphological plasticity warrants dedicated conservation efforts and additional taxonomic scrutiny.

Gongolaria elegans and *G. montagnei*, two other polymorphic entities more closely related to *G. nodicaulis*, were confirmed from Almería (Spain) westwards to at least Catalonia and Menorca, respectively. Still, they are presumed, based on bibliographic records, to be more widely distributed in at least the western Mediterranean basin. *Gongolaria elegans* s.l. comprised two very distinct morphotypes, informally named 'shallow' and 'deep' to reflect their typical depth preferences. The latter, known from around the Medes Islands (NW Mediterranean), is undergoing a regional range expansion (Medrano *et al.*, 2020). As in deeper *G. rayssiae* (Mulas *et al.*, 2020), its fronds are much thicker, spiny and blueish compared with the typical shallow morphotype (see also Mariani *et al.*, 2019). These differences are presumed to reflect greater depth and exposure, but we cannot exclude cryptic differentiation not captured by *cox*1 alone. The same applies to Spanish *G. sauvageauana*. Overwintering individuals from Columbretes (Balearic Sea) shared the same *cox*1 sequence with *G. elegans*. The existence of intermediate forms of dubious assignment between both taxa (as observed by RB) is in line with a very close relationship, or even conspecificity. Genetic data from another Spanish (Alicante) population (Jódar-Pérez *et al.*, 2020) also place *G. sauvageauana* among tophulose *Gongolaria*, but the marker employed (23S-tRNA-Lys spacer (mtIGS)) cannot be directly compared. Additional samples of this important Mediterranean taxon, preferably from other Mediterranean regions, are necessary to confirm the geographic consistency of this taxon and its genetic affinity with *G. elegans*. *Gongolaria montagnei* s.l. MOTU comprised a diversified collection of tophulose algae from shallow and deeper (−20 m) waters, including some identified as *G. algeriensis* (Sales & Ballesteros, 2009; Sousa *et al.*, 2019a; Jódar-Pérez *et al.*, 2020), depending on tophule ornamentation. In Almería (Cabo de Gata) and Alicante (Santa Pola) spinose algae with smooth tophules occurred in mixed stands with *Ericaria amentacea*. In Menorca, smoother algae from mostly sheltered bays and possessing either spiny or smooth tophules produced the same *cox*1 sequence that was also shared with deeper water collections collected throughout the Balearic Sea. These data suggest a single, very polymorphic species, and imply that ornamentation of tophules can have less diagnostic significance than conventionally assumed (see Serio, 1995).

Somewhat unexpectedly, collections from the central (Sicily) and eastern (Crete) Mediterranean previously identified as *G. elegans* (Draisma *et al.*, 2010), or identified by local divers as *Cystoseira spinosa* (= *G. montagnei*), were genetically (but also morphologically) distinct from these more western taxa. These algae were characterized by prominent cauloid apexes, at least during summer, but were rather plastic with respect to depth (large rock-pools to at least a few metres) and exposure. *Gongolaria montagnei* and *G. squarrosa* (De Notaris) Kuntze from Croatia and Draisma's *C. elegans* (Sicily) showed similar 23S-tRNA-Lys spacer (mtIGS) sequences (Rožić *et al.*, 2012; Sousa *et al.*, 2019a), that differed from Iberian *G. montagnei*. These data strongly suggest that those samples also correspond to this species, confirming its presence in the Adriatic and suggesting a ubiquitous distribution in the eastern and central Mediterranean. Until the correct name for this taxon becomes clearer, this entity is conservatively named as *Gongolaria* sp. 2, but a new name might become necessary.

Strengths and shortcomings of the barcoding approach

Straightforward identification of species from single-marker sequence data is appealing but needs to be critically interpreted (Schindel & Miller, 2005; Collins

& Cruickshank, 2013). It remains to be demonstrated that inferred *cox*1 MOTUs match, without many exceptions, 'real' biological species, something that requires independent nuclear data, but also the input of non-molecular taxonomy (Schander & Willassen, 2005). The adopted method for MOTU identification, based on haplogroup (dis)continuities rather than divergence thresholds, or more complex species delimitation methods, is subjective and open to criticism. *Cox*1 variation may also be failing to capture all the biological diversity within shallow species complexes, such as among the *E. crinita* s.l. and tophulose *Gongolaria* complexes. Globally, most MOTUs represented relatively consistent morphological and/or biogeographic entities. However, there were several exceptions (e.g. *Gongolaria* sp. 1, *Ericaria selaginoides* haplogroups A–C), and, a posteriori, many remain quite difficult to define and recognize based on morphology alone. Finally, organelle genomes are especially prone to introgression, and hybridization is known to have permeated the evolution of other fucoid radiations (Coyer *et al.*, 2002; Neiva *et al.*, 2010, 2017). Given the frequent spatial co-existence or proximity of related *Cystoseira*, *Ericaria* and *Gongolaria* species, opportunities to hybridize presumably abound, and mtDNA capture (and even allopolyploidy) may represent an overlooked but important factor confounding true species boundaries and affinities. Bearing all these potential pitfalls in mind, the preliminary (but comprehensive) *cox*1-based species hypotheses advanced above provide a good starting point for future scrutiny using more integrated approaches.

*Cox*1 polymorphism was genus-specific, but a small overlap between intra- and interspecific divergence was observed in all three genera. These overlaps can only be, even if very marginally, underestimated, since more intra-specific diversity is likely to exist beyond what was captured in the relatively low number of samples analysed. The absence of true barcoding gaps is the best explanation for the conservatism of model-based species delimitation methods, since intraspecific variation and (putative) sister species are mixed on the short terminal branches, and thus difficult to discriminate (Lowenstein *et al.*, 2009). Despite the poor performance and limited use of *cox*1 barcodes to assist algorithm-based species delimitation, the barcodes remain useful to assist species identifications. Even in the cases where divergence between MOTUs was very shallow, such as the tophulose complex comprising *G. elegans* s.l., *G. montagnei* s.l., *G. nodicaulis*, *G. gibraltarica* and *Gongolaria* sp. 2, the few mutations were species-specific and thus diagnostic. One of the major contributions of this study is precisely making available a library of voucher-backed *cox*1 barcodes for all four 27 MOTUs recognized,

including, for many, multiple haplotypes covering part of species intra-specific variation. Predictably, this comprehensive library will allow confirming or assign dubious samples to recognized MOTUs, or otherwise recognize new entities, using blasting algorithms and/or by direct comparison with published sequences. Misidentifications (bearing extensive introgression) are unlikely, since most major species were included and replicated, i.e. identifications were not based on single 'types' and/or distinct marker sets. This effort is relevant since identification errors can propagate throughout online databases (e.g. GenBank, see Fort *et al.*, 2021), just like morphological misidentifications are propagated via literature citations and species check-lists. *Gongolaria mauritanica* provides perhaps the most illustrative example. Only after analysing, for the same marker, populations from Gibraltar, Canary Islands and Mauritania did it become apparent that this taxon comprised three distinct entities and could be partially synonymized with *G. nodicaulis*, despite the available taxonomic keys, verifiable vouchers and two recent genetic studies (Orellana *et al.*, 2019; Sousa *et al.*, 2019*a*).

Although not the primary goal of the study, the 'few taxa, few genes' phylogenetic approach employed offered more resolution to reconstruct species relationships than previous studies. Owing to the more diverse and pre-screened panel of species included, and the longer concatenated alignment used, this was expected. Ultimately, however, it added few new insights when compared with *cox*1 data alone, as relationships between several lineages and shallow species complexes were poorly supported. These data suggest that *cox*1 alone can provide a good first proxy to reconstruct broad phylogenetic patterns and species affinities, and that adding more plastid genes may only marginally increase phylogenetic signal. Taking into consideration this limitation, the seemingly recent radiation of many species' complexes and the potentially confounding effects of past and ongoing hybridization, other approaches beyond (but building on) this classic mtDNA-based approach (e.g. genomic data, see below) seem unavoidable to clarify the evolution of these genera.

Emerging patterns and future directions

This baseline study raises more questions than answers and can be expanded along multiple fronts. First, screening more regional floras for the same cost-effective barcoding marker will contribute to refine MOTUs range limits and, most likely, lead to new taxonomic and biogeographic insights. Vast areas of the Mediterranean remain unexplored, but genetic assessments are missing also for regions for which good (morpho)species baselines exist (e.g.

much of the Ligurian, Tyrrhenian, Adriatic, Ionian, Aegean and the Levantine Seas, Algeria to Egypt). Such data will be critical to clarify the validity and affinities of unsampled/ poorly sampled taxa (e.g. *G. sauvageauana*, *G. susanensis*, *C. crinitophylla*, *C. schiffneri* Hamel, *C. senegalensis*, *E. funkii* (Gerloff & Nizamuddin) Molinari & Guiry, *C. jabukae* Ercegovic), but also emerging patterns of diversity and species assembly. For instance, the (still limited) phylogeographic data available seem to suggest some complementarity in the distributions of some cryptic/sibling Mediterranean taxa, with *E. brachycarpa* s.s., *E. selaginoides* haplogroup C and *Gongolaria* sp. 2 apparently replacing in the eastern basin the more western *E. balearica*, *E. selaginoides* haplogroups A and B, and *G. montagnei*/*G. elegans*. Regional contacts were apparent along the broader Pantelleria/Sicily/Malta axis, but the extent of range overlaps in the central Mediterranean remain undetermined. Such areas provide ideal geographic settings to investigate vicariance/secondary contact dynamics of western and eastern floras. *Cox*1 data also revealed high levels of structuration among *Cystoseira* s.s. species and *Ericaria selaginoides* complex, with Macaronesian populations showing high levels of haplotypic diversity and/or endemism. Future studies may confirm the suspected refugial role of these archipelagos within the broader Atlantic and their apparent isolation with respect to core Mediterranean/Atlantic ranges.

Secondly, new markers and approaches should be employed to resolve persistent taxonomic issues and elucidate the evolution of this rich North-east Atlantic endemic flora. Nuclear gene data are crucial to validate species boundaries, but most traditional markers (ITS, LSU) are unlikely to have sufficient resolution to differentiate closely related taxa (Coyer *et al.*, 2006; Phillips *et al.*, 2008; Silberfeld *et al.*, 2010). Gene-flow markers (e.g. microsatellites or RADseq) can be used to assess the degree of reproductive isolation of cryptic *E. selaginoides* haplogroups, *E. crinita* s.l. and tophulose *Gongolaria* spp., particularly wherever conflicting MOTUs/morphotaxa co-occur or overlap their ranges. Genome-wide approaches offer another powerful alternative to resolve species relationships and detect inter-specific gene-flow and introgression (Sousa *et al.*, 2019*b*; Bringloe *et al.*, 2021). These methods are considerably more expensive, but increasingly seem sensible, since candidate species and populations of interest are now a priori much better circumscribed. Coupled with better biogeographic data, they can also be employed to investigate the diversification of these genera at multiple timescales, including for example the evolutionary dynamics in and out of the Mediterranean (Le Gall *et al.*, 2021). Phylogeographic data identified multiple sister species with disjointed

(or nearly so) Atlantic vs Mediterranean ranges, including *Cystoseira humilis*/*C. pustulata*, *Ericaria selaginoides* A/A, B, C, *Gongolaria* sp. 1/*G. rayssiae*, and *G. nodicaulis*/*Gongolaria* spp., which represent good models to examine recent back and forth migrations, and niche shifts in face of very shallow differentiation.

Finally, the development of new taxonomic keys reflecting more closely the genetic-based circumscriptions of species is also warranted. *Cox*1 barcoding/phylogeographic data resulted in the synonymy of two taxa (*G. mauritanica* sensu Sauvageau, *C. aurantia* sensu Orellana *et al.*, 2019) and the recognition of five new taxa (*Cystoseira pustulata*, *E. balearica*, *Gongolaria* sp. 1, *G. gibraltarica* and *Gongolaria* sp. 2), in part associated with narrower circumscriptions of at least five related species (*C. compressa* s.s., *C. humilis* s.s., *E. brachycarpa* s. s., *G. elegans* and *G. montagnei*). On the other hand, *E. crinita* s.l. and *E. selaginoides* haplogroup A lumped multiple taxa, whereas *G. montagnei* s.l. apparently incorporated another taxon (*G. algeriensis*) but did not include parts of its older range (currently assignable to *Gongolaria* sp. 2). Identifying sets of characters that are universal (or nearly so) and unique to each MOTUs is beyond the scope of the present study, but is anticipated to be, as ever, a very challenging task. A few species are morphologically and geographically well delimited (*E. dubia*, *E. sedoides*, *E. zosteroides*, *G. baccata*). However, the majority of taxa shows considerable intraspecific plasticity and potential for confusion with related (and sometimes not so related) cryptic species. Significantly, several key characters traditionally used in *Cystoseira* s.l. taxonomy seem to be more variable and bear less taxonomic value than conventionally assumed. Examples include, as discussed above, variation in the spinosity of cauloid apexes (a key trait to discriminate, for example, *E. crinita* from *E. barbatula*), the ornamentation of tophules (a key trait to discriminate among *Gongolaria* spp.) and the habit type (a trait used to discriminate morpho-taxa of the *E. selaginoides* complex), in all cases showing little correspondence with inferred MOTUs. Some variation may be region-specific and thus easier to account for. For instance, only the northernmost Atlantic populations of *C. foeniculacea* seem to lack densely covered spiny cauloids, an otherwise reliable character to identify this very polymorphic species. On the other hand, cryptic genetic entities among the *Ericaria selaginoides* and tophulose *Gongolaria* complexes appear to have an appreciable degree of spatial structuration, so that tentative identifications (or elimination) may prove in some cases easier if based on geography than morphology. Whichever the case, practical criteria are essential to assist species identifications in the field.

Cystoseira s.l. forests have been declining in recent years. In a scenario of ongoing climatic change, further changes in the range and abundance of *Cystoseira* s.l. seem inevitable and, given their role as foundational species, are likely to have substantial negative ecological effects. Noticeably, some species seem particularly vulnerable due to their narrow distributions, whereas others, as relict members of divergent phylogenetic lineages, also bear special conservation value. Considering the low recovery potential of many species, reforestation actions have been increasingly employed to accelerate the recovery of littoral ecosystems (reviewed in Cebrian *et al.*, 2021). Given taxonomic uncertainty and the phylogeographic structure observed in some taxa (see also Bermejo *et al.*, 2018) molecular pre-screening should be encouraged. As our understanding regarding species boundaries, ranges and affinities improves, so will our ability to recognize, anticipate and eventually manage cryptic diversity losses in this unique flora endemic to the North-east Atlantic and the Mediterranean.

Taxonomic and nomenclatural proposals

Cystoseira C.Agardh, 1820, *nom. cons.*

Cystoseira foeniculacea f. *formosensis*, Neiva & Serrão, forma nov. (Fig. 8a)

DIAGNOSIS: Diffuse, free-living with extremely simplified morphology, lacking anchoring holdfasts and true cauloids. Fronds with a divaricate branching pattern along a single plane, with compressed (elliptical in cross section) axes that break very easily above a certain size. Fronds and axes smooth, without spiny appendages. Presumed only to propagate clonally, as no reproductive structures have been found.

TYPE LOCALITY: Armona Island (Ria Formosa), Portugal.

HOLOTYPE: ALGU A 3928, 14 JUN 2018.

ISOTYPE: ALGU A 3929, We are not providing paratypes.

ETYMOLOGY: *formosensis*, an adjective, derived from *formosa*, and *-ensis*, referring to living in; a reference to type locality, the Ria Formosa Lagoon.

HABITAT: Coastal lagoon, from spring low-tide limit to a few metres depth, often in patches and edges of seagrass meadows.

DISTRIBUTION: So far only known from the type locality.

Cystoseira pustulata (Ercegovic) Neiva & Serrão, comb. nov.

BASIONYM: *Cystoseira abrotanifolia* subsp. *pustulata* Ercegovic *Fauna et Flora Adriatica* Vol 2: 113, pls XXX, XIV e, g. 1952.

HOMOTYPIC SYNONYM: *Cystoseira compressa* subsp. *pustulata* (Ercegovic) Verlaque 2015: 219.

TYPE LOCALITY: E. Adriatic Sea.

NOTES: Elevated to species rank based on molecular data. In the literature it is often confused with *Cystoseira compressa* (Esper) Gerloff & Nizamuddin 1975 and *Cystoseira humilis* Schousboe ex Kützing 1860. Representative cox1 sequences: OK480303, OK480323.

Ericaria Stackhouse 1809

Ericaria dubia (Valiante) Neiva & Serrão, comb. nov.

BASIONYM: *Cystoseira dubia* Valiante *Fauna und Flora des Golfes von Neapel und der angrenzenden Meeresabschnitte* 7: 24, pl. XV, 1883.

TYPE LOCALITY: Gulf of Naples.

NOTES: Assigned to genus *Ericaria* based on molecular data. Representative *cox*1 sequence: OK480325.

Ericaria sedoides (Desfontaines) Neiva & Serrão, comb. nov.

BASIONYM: *Fucus sedoides* Desfontaines *Flora atlantica* 423, pl. 260, 1799.

HOMOTYPIC SYNONYM: *Cystoseira sedoides* (Desfontaines) C.Agardh 1820: 53.

TYPE LOCALITY: 'in fundo maris … prope La Calle' [El Kala, Algeria].

NOTES: Assigned to genus *Ericaria* based on molecular data. Representative *cox*1 sequence: OK480330.

Ericaria corniculata (Turner) Neiva & Serrão, comb. nov.

BASIONYM: *Fucus ericoides* var. *corniculatus* Turner *Fuci* Vol. III: 132,135, 1809–1811 (?).

HOMOTYPIC SYNONYM: *Cystoseira corniculata* (Turner) Zanardini 1841: 243.

LECTOTYPE LOCALITY (here designated): Adriatic Sea.

NOTES: The dates of publication of the parts of Turner's *Fuci* are uncertain as no copies exist in the original papers in which the plates were issued periodically. Turner (1809–1911: 132) described material of this species from the Adriatic and Sri Lanka (the latter not considered here). Assigned to genus *Ericaria* based on molecular data. Representative *cox*1 sequences: OK480406, OK480407.

Ericaria balearica (Sauvageau) Neiva, Ballesteros & Serrão, comb. nov.

BASIONYM: *Cystoseira balearica* Sauvageau, *Bulletin de la Station biologique d'Arcachon* 14: 390, 528, 1912.

HOMOTYPIC SYNONYM: *Cystoseira brachycarpa* var. *balearica* (Sauvageau) Giaccone (in Ribera *et al.*, 1992: 124).

TYPE LOCALITY: Mallorca, Balearic Islands.

NOTES: Elevated to species rank based on molecular data. Previously within *Ericaria brachycarpa* (J. Agardh) Molinari & Guiry, 2020. Representative *cox*1 sequences: OK480393, OK480399. The synonymy of *Cystoseira caespitosa* Sauvageau 1912: 223, 526 (type locality: Banyuls-sur-Mer) with this taxon requires genetic verification.

Gongolaria Boehmer 1760
Gongolaria sonderi (Kützing) Neiva, João Soares & Serrão, comb. nov.

BASIONYM: *Treptacantha sonderi* Kützing *Tabulae phycologicae*, Vol. 11, pl. 28: fig. III, 1860.
HOMOTYPIC SYNONYM: *Cystoseira sonderi* (Kützing) Piccone 1886: 41.
TYPE LOCALITY: Cape Verde.
NOTES: Assigned to genus *Gongolaria* based on molecular data. Representative *cox*1 sequence: OK480425.

Gongolaria gibraltarica (Sauvageau) Neiva, Bermejo & Serrão, comb. nov.

BASIONYM: *Cystoseira selaginoides* var. *gibraltarica* Sauvageau *Bulletin de la Station Biologique d'Arcachon* 17: 31, 1920.
HOMOTYPIC SYNONYM: *Cystoseira gibraltarica* (Sauvageau) P.J.L.Dangeard 1949: 128, 133, no fig.
LECTOTYPE LOCALITY: Algeciras, Spain.
NOTES: Elevated to species rank based on molecular data. Traditionally identified as *Gongolaria mauritanica* (Sauvageau) Molinari & Guiry, 2020. Representative *cox*1 sequence: OK480491.

Gongolaria gibraltarica f. lacunarum Neiva & Serrão, forma nov. (Fig. 8b).

DIAGNOSIS: Free-living alga with extremely simplified morphology, lacking anchoring holdfasts and cauloids. Diffuse growth, primary branches cylindrical, to 40 cm, 1–2 mm diameter, higher-order branches arranged in multiple directions and normally making acute angles with main axes. Fronds and axes smooth, without spinose or foliose appendages. Aerocysts often present, solitary or arranged in chains. Presumed to proliferate clonally by fragmentation, as no reproductive structures have been observed.
TYPE LOCALITY: Culatra Island (Ria Formosa), Portugal.
HOLOTYPE: ALGU A 4120, 29 JUL 2018.
ISOTYPE: ALGU A 4121.
ETYMOLOGY: *lacunarum*, a plural genitive noun (feminine) in apposition, meaning of the lakes or lagoons; a reference to its habitat, coastal lagoons.
HABITAT: Coastal lagoons, from spring low-tide limit to a few metres depth, often growing in seagrass and *Caulerpa prolifera* meadows.

DISTRIBUTION: Coastal lagoons of southern Portugal, Gulf of Cadiz and N Morocco, possibly wider.
NOTES: Traditionally identified as the unrelated *Gongolaria barbata* f. *repens* (A.D.Zinova & Kalugina) Sadogurska 2021 (≡ *Cystoseira barbata* f. *repens* (*A.D.Zinova* & *Kalugina*)), reclassified based on molecular data. Representative *cox*1 sequence: OK480503.

Supplementary material

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at https://doi.org/10.1080/09670262.2022.2126894
Supplementary table S1. PCR amplification conditions.
Supplementary table S2. List of *cox*1 sequences of *Cystoseira, Ericaria* and *Gongolaria* spp. from Genbank used in the present study.
Supplementary table S3. Submitted Genbank sequences and data-sets (last four columns) used in the different analyses.

Author contributions

JN, GAP, EAS conceived the study; JN, RB, AM, PC, DMF, EB, BS, DS, EN, JS, JV, MM, SSS, GAP, EAS collected the samples; JN, MM, SSS obtained sequences and conducted genetic analyses; MDG conducted the taxonomic review; JN drafted the manuscript with important contributions from RB, EB, DS, FT, SSS, MDG and EAS. JN, PA, EB, BS, DS, FT, AI, EAS provided funds for sampling campaigns and/or laboratory analyses. All authors read, edited, and approved the final manuscript.

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