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Molecular identification of the brown algae, *Cystoseira* spp. (Phaeophycae, Fucales) from the Adriatic Sea – preliminary results

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In the attempt to identify an appropriate molecular marker which will enable genetic distinction between different Cystoseira *species from the Adriatic Sea, two mitochondrial molecular markers were tested: the mt 23S rDNA and the mt23S-tRNAVal spacer. Two species were studied:* Cystoseira spinosa *and* Cystoseira squarrosa. *Sequence analyses showed no variation in the mt 23S rDNA among all individuals analyzed. But the analysis of the mt23S-tRNAVal spacer showed a differentiation between three haplotypes named A, B and C. The most abundant haplotype A was found in equal number in both species, while haplotype B was found only in* C. spinosa *and haplotype C was found only in* C. squarrosa. *However, when comparing to sequences available for several selected Mediterranean* Cystoseira *species, the mt23S-tRNAVal spacer failed to discriminate between species. Although these results indicate a limited use of the mitochondrial mt23S-tRNAVal intergenic spacer for discrimination among Adriatic* Cystoseira *species, they could also be interpreted as a sign of conspecificity of the investigated species or the reflection of a recent radiation. Further analysis will be necessary to improve molecular identification of these brown algae.*

Key words: *Cystoseira*, Adriatic Sea, mitochondrial DNA, molecular markers, molecular phylogeny

INTRODUCTION

The brown algae of the genus *Cystoseira* are among the most dominant and ecologically important species in the Mediterranean and the Adriatic Sea. This genus inhabits rocky substrates of the infralittoral zone where they play an important ecological role in providing habitat, food and shelter as well as spawning and nursery grounds for a wide variety of organisms (GOMEZ GARETTA, 2000; reviewed in DRAISMA *et al.* 2010). However, in recent years, *Cystoseira* stands have been significantly reduced or disappeared due to habitat destruction, overgrazing and eutrophication in the Mediterranean Sea (THIBAUT *et al.,* 2005; SUSINI *et al.,* 2007a and b; MANGIALAJO *et al.,* 2007, 2008). Despite its ecological importance, the taxonomy of species within the genus is still poorly understood and no studies have yet explored the genetic diversity of *Cystoseira* species in the Adriatic Sea.

The genus accounts for a large number of taxa including species, varieties and forms, currently 56 in the Adriatic Sea (GUIRY & GUIRY, 2012), 348 names total recorded in the Index Nominum Algarum (SILVA, 2012.), 292 names of which 38 have been flagged as currently accepted taxonomically (GUIRY & GUIRY, 2012), which may exhibit great adaptability to different ecological conditions, mostly by morphological variation (ERCEGOVIĆ, 1952, 1959; GOMEZ GARRETA, 2000). It is a common occurrence that due to those adaptations, the ecomorphs differ greatly from the average morphology of the species, that makes difficult to distinguish which morphological variation is an adaptation to ecological conditions, and which is a characteristic of a different species. Species determination is made even more difficult by the lack of comprehensive identification keys for the genus. The only currently available key for the Adriatic *Cystoseira* species (ERCEGOVIĆ, 1952) based on morphology is outdated and was written in a period when sampling possibilities, and thus also determination, were limited. Difficulties arise while identifying the species, as several names may have been applied to the various populations or ecomorphs belonging to a single species, i.e. the morphological variability of the species has been overlooked. The situation is similar for other Sargassaceae genera such as for example the genus *Sargassum* (MATTIO & PAYRI, 2011). Since the *Cystoseira* genus is in an active process of speciation (ERCEGOVIĆ, 1952; CORMACI *et al.*, 1992), the current method of determining relations within the genus based primarily on morphological characteristics, is shown as being insufficient to unambiguously separate some species of this genus. Because of this, the focus is increasingly shifting to molecular methods of studying marine algae phylogeny, particularly brown algae, and this genus. In order to overcome morphological ambiguities in the identification of speceis, molecular markers are nowadays used (JEGOU *et al.,* 2010).

Several different molecular markers have been used in recent studies of brown algae molecular taxonomy and phylogeny. The most common ones are nuclear ribosomal markers, ITS, SSU and LSU, (ROUSSEAU *et al.,* 2001; LE CLERC *et al.,* 1998; HARVEY & GOFF, 2006.), ITS2 (JEGOU *et al.,* 2010), plastid markers, rbcL and psaA (BITTNER *et al.,* 2008; CHO *et al.,* 2006), and mitochondrial markers, mt 23S and mt spacer (COYER *et al.,* 2006; DRAISMA *et al.,* 2010). Hovwever, most of these markers have been used for delineation among the higher taxonomic units such as genera and families and rarely for distinguishing between the species. This is especially the case in the order Fucales, the family Sargassaceae, and the genus *Cystoseira,* which require highly variable markers to determine relations between the taxa (DE REVIERS *et al*., 2007). SUSINI *et al.* (2007b) have noted high genetic variations in the populations of the genus *Cystoseira*, and suggest that due to those variations the populations should be considered separately when studying the ecology of the genus.

Recently, DRAISMA *et al.* (2010) have underlined the usefulness of the mitochondrial 23S in the delineation of Sargassaceae genera and the potential of the mt23S-tRNAVal spacer at below genus level. With the purpose of identifying the appropriate molecular marker with which species distinction of the Adriatic *Cystoseira* could be achieved, we choose to test those two mitochondrial molecular markers in a preliminary study. Two Adriatic *Cystoseira* species were analyzed: *Cystoseira spinosa* (Savageau, 1912) and *Cystoseira squarrosa* (De Notaris, 1841). The data was then compared to published sequences of other Mediterranean *Cystoseira* species; *C. bracata*, *C. spinosa, C. susanensis, C. elegans, C. baccata* and *C. usneoides* (from DRAISMA *et al*., 2010).

MATERIAL AND METHODS

Sample collection and DNA preparation

Ten individuals of each species were sampled from the rocky bottom between 3 and 5 m of depth. C*ystoseira spinosa* was collected from the island of Brač (43°23'23.52"N -

Table 1. Primers, PCR conditions and reaction ingredients

Gene region	Primer	Primer sequence (5' – 3')	PCR reaction ingredients (in 20 µl)	PCR Cycling conditions
Mt 23S rDNA	mt23S –FB (Draisma et al, 2010) mt23S – RB (Draisma et al, 2010)	AGCGTAACAGCTCACTGACCTA CTGTGGCGGTTTAAGGTACGGTT	2 µl 1 x PCR buffer (Invitrogen) 1.2 µl 15 mM MgCl2 (Invitrogen) 2 µl of each dNTP (2mM) 1 µl of each primer (10mM) 0,2 µl *Taq* polymerase (Sigma) 1 µl DNA template (concentration undetermined)	(1) 2 min at 94°C (2) 40 cycles of 30s at 94°C, 30s at 50 °C and 40s at 72 °C (3) final extension for 5 min at 72 °C (Coyer et al, 2006)
Mt spacer (mt23S-tRNA Lys)	tRNALys – F1 (Draisma et al, 2010) tRNALys – R1 (Draisma et al, 2010)	GGGGTGAAAAATATCACTTTGA AACCCAAGACCCTCGGATTA	2 µl 1 x PCR buffer (contains 15mM MgCl2, Sigma) 2 µl of each dNTP (2mM) 1 µl of each primer (10mM) 0,2 µl *Taq* polymerase (Sigma) 1 µl DNA template (concentration undetermined)	(1) 2 min at 94°C (2) 40 cycles of 30s at 94°C, 1 min at 50°C and 1 min at 72°C (3) final extension for 5 min at 72 °C (Coyer et al, 2006)

16°27'34.78"E) in March 2010, and *C. squarrosa* from the Dubrovnik city area (42°35'13.45"N - 18°10'35.86"E) in July 2010. Morphological analysis was guided with characteristics described by ERCEGOVIĆ (1952). Immediately after collection, a part of the branch least overgrown by epiphytes was separated from each specimen. Visible epiphytes were then mechanically removed from the sampled part, and it was washed with distilled water and stored in silica gel. The rest of the talus was stored in 4% formaldehyde. Total DNA was isolated from silica dried algal tissue using Qiagen Plant mini kit and additionally purified using Qiagen Plasmid mini kit.

PCR primers, PCR and sequencing

Primers published by DRAISMA *et al.* (2010) for the mt 23S and the mt23S-tRNAVal spacer were used for PCR amplification and sequencing. Primers, PCR conditions and reaction ingredients are listed in Table 1. The thermo cycler used in PCR amplification of all regions was Applied Biosystems 2720. The amplified DNA fragments were purified using QIAEX II gel extraction kit 150 (Qiagen). Sequencing was done by Macrogen in Korea using an ABI PRISM 3100 Avant Genetic Analyzer.

Table 2. Species, localities, specimens and GenBank accession numbers for sequence data generated in this study

Species	Collection site	Specimens	Sequence	Haplotypes	Accession number
Cystoseira spinosa	Brač Island	C_spinosa_1 C_spinosa_4	23S rRNA, mitochondrial	Haplotype 1	HQ438490
Cystoseira squarrosa	Dubrovnik city area	C_squarrosa_1 C_squarrosa_3	23S rRNA, mitochondrial	Haplotype 1	HQ438491
Cystoseira spinosa	Brač Island	C_spinosa_1 C_spinosa_2 C_spinosa_4 C_spinosa_6 C_spinosa_10	23S ribosomal RNA – tRNA Val intergenic spacer, mitochondrial	Haplotype A	HQ438492
		C_spinosa_3 C_spinosa_5 C_spinosa_7	23S ribosomal RNA – tRNA Val intergenic spacer, mitochondrial	Haplotype B	HQ438493
Cystoseira squarrosa,	Dubrovnik city area	C_squarrosa_3 C_squarrosa_4 C_squarrosa_5 C_squarrosa_6 C_squarrosa_8 C_squarrosa_9	23S ribosomal RNA – tRNA Val intergenic spacer, mitochondrial	Haplotype A	HQ438494
		C_squarrosa_1	23S ribosomal RNA – tRNA Val intergenic spacer, mitochondrial	Haplotype C	HQ438495

Data analysis

Raw sequences were edited and trimmed manually using Bioedit v. 7.0.9. (HALL, 1999). The number of polymorphic sites and phylogenetically informative sites were determined using the DnaSP v5 (LIBRADO & ROZAS, 2009). Alignment of sequences was processed using ClustalX (LARKIN *et al*., 2007) and visualized in GeneDoc (NICOLAS *et al.* 1997) for each marker independently. A total of 11 sequences for other Mediterranean *Cystoseira* species available on GenBank and published by DRAISMA *et al.* (2010) were included in the analyses. The most suitable model of nucleotide evolution was determined by the Akaike Information Criterion, AIC, (AKAIKE, 1974) as implemented in JModelTest (POSADA, 2008): for the 23S rDNA the selected model was TrN+G with gamma=0.013, and for the mt spacer the selected model was HKY, with ti/tv=1.4910. Bayesian analyses were performed using MrBayes 3.1.1 (HUELSENBECK & RONQUIST, 2001). The parameters of the Markov Chain Monte Carlo (MCMC) analysis comprised two runs (four chains each) for 500000 generations, with the sample frequency set to 100 and discarding first 1250 trees as the burn in. Thus, the posterior probabilities of the clades were determined from 3750 trees and the 50% majority-rule consensus tree was built.

RESULTS AND DISCUSSION

A total of four sequences for the mt23S rDNA (394 pb) and 15 sequences for the mt intergenic spacer (mt23S-tRNAVal) (333 pb) were successfully amplified and sequenced. Sequences of 23S rDNA were obtained for two individuals of *C. spinosa* and two individuals of *C. squarrosa*. Sequences of mt spacer were obtained for eight individuals of *C. spinosa* and seven individuals of *C. squarrosa* (Table 2). The sequences were deposited in GenBank (Table 2).

The 23S rDNA sequences showed no variability in all four investigated specimens (data not shown). This result indicated that the mt23S marker is not suitable for distinguishing between *C. spinosa* and *C. squarrosa* and wasn't further analyzed.

The aligned sequences for the intergenic spacer included 18bp of the 3'end of the 23S gene to the tRNA Val gene, encompassing also the tRNA Lys gene at the 5'end of the tRNA Val gene (COYER *et al*., 2006). The mt23S-tRNAVal intergenic spacer sequences of eight specimens of *C. spinosa* and seven specimens of *C. squarrosa* revealed two informative sites, one of them being parsimony informative, and the other being singleton variable site. The number of haplotypes was three, named A, B, and C (Fig. 1). The most frequent haplotype was

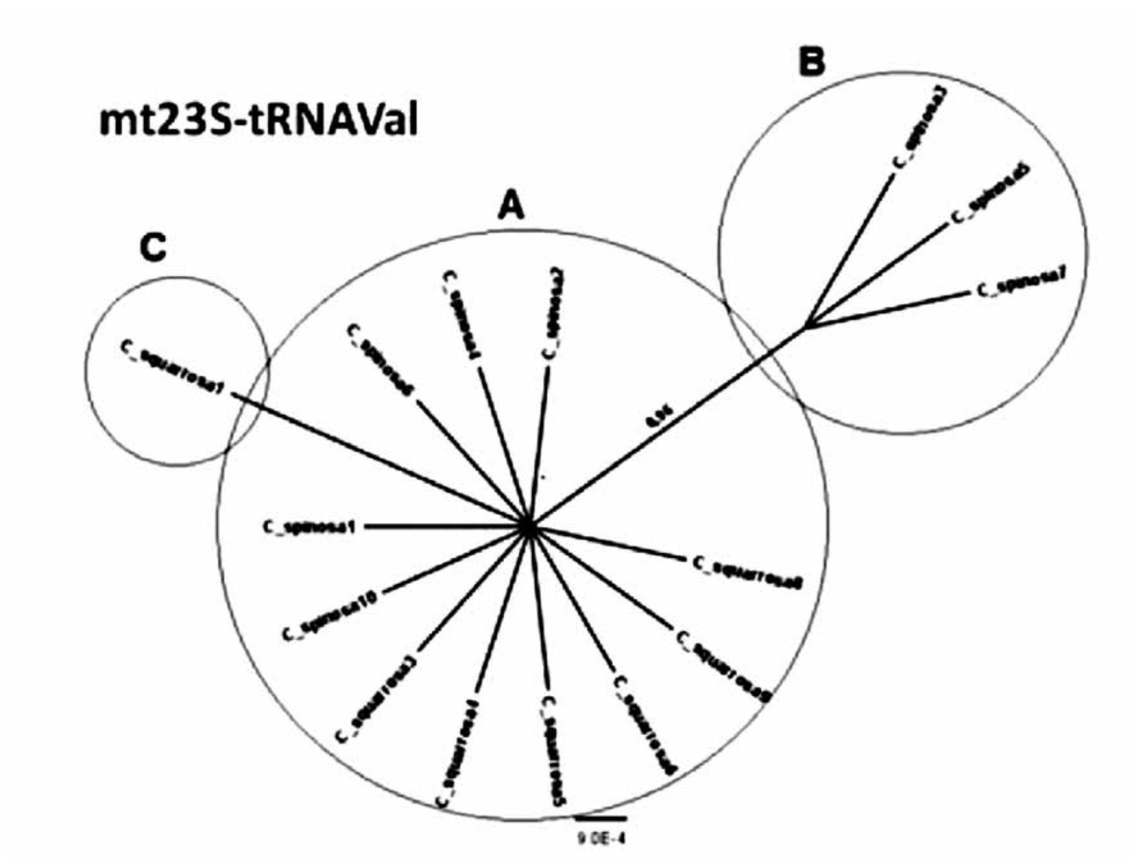

Fig. 1. Radial tree showing relations between haplotypes A, B and C for the mt spacer (mt23S-tRNA Val) of C. spinosa and C. squarrosa *using Bayesian analysis. Number on the branch is posterior probability. Scale indicates expected number of substitutions per site*

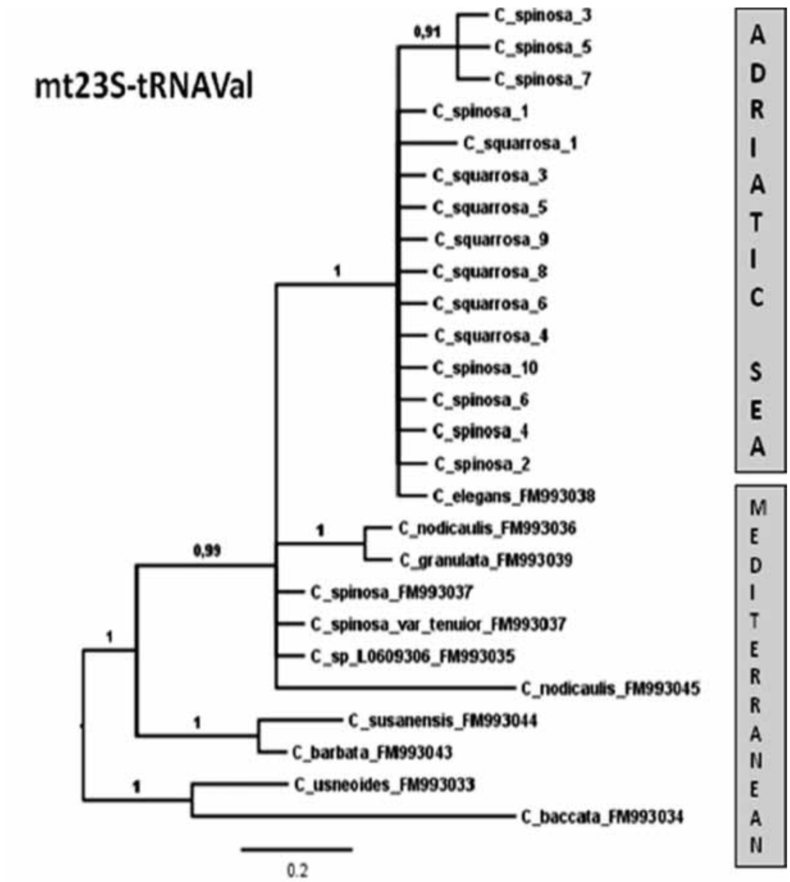

Fig. 2. Phylogenetic tree of the mt23S-tRNAVal haplotypes of the Adriatic C. squarrosa *and* C. spinosa *and Mediterranean Cystoseira speciesis (taken from DRAISMA et al., 2010) (*C. bracata, C. spinosa, C. susanensis, C. elegans, C. baccata and C. usneoides .C. nodicaulis, C. granulata*). The tree was constructed using Bayesian analysis. Numbers on nodes are posterior probabilities. Scale indicates expected number of substitutions per site*

haplotype A. It was found in five *C. spinosa* and six *C. squarrosa* specimens. The haplotype B was found in three *C. spinosa* specimens, whereas the haplotype C was found only in one *C. squarrosa* specimen. The grouping of a large number of samples of both species in the haplotype A indicates that the mt23S-tRNAVal spacer is insufficiently informative for distinction between *C. spinosa* and *C. squarrosa.*

This conclusion is further confirmed by comparing the mt23S-tRNAVal intergenic spacer sequences of *C. spinosa* and *C. squarrosa* to the other Mediterranean *Cystoseira* species published by DRAISMA *et al.* (2010). The final mt23S-tRNAVal dataset covered 26 sequences; 15 sequences of Adriatic *Cystoseira* species and 11 sequences dowloaded from the GenBank, which exhibited the highested sequence homology to the Adriatic *Cystoseira* species. The topology of the tree shows grouping of both *C. spinosa* and *C. squarrosa* specimens into a single well suported clade (posterior probability= 01). Interestingly, *C. elegans* from Sicily clustered also into this clade, whereas several *C. spinosa* sequences originating from Spain clustered separately (Figs. 2 and 3).

The preliminary results indicate some variability of the mt intergenic spacer (mt23S-tRNA Val) among species and populations of the two species studied. However, the variability of this region is insufficient to achieve species separation among the analyzed Adriatic *Cystoseira* specimens. It is possible, that the analyzed Adriatic species of *Cystosiera* are highly related species with a relative young origin, where genetic mutations haven't had the time to accumulate. Alternatively, the two populations studied would belong to a single species and not two as origi-

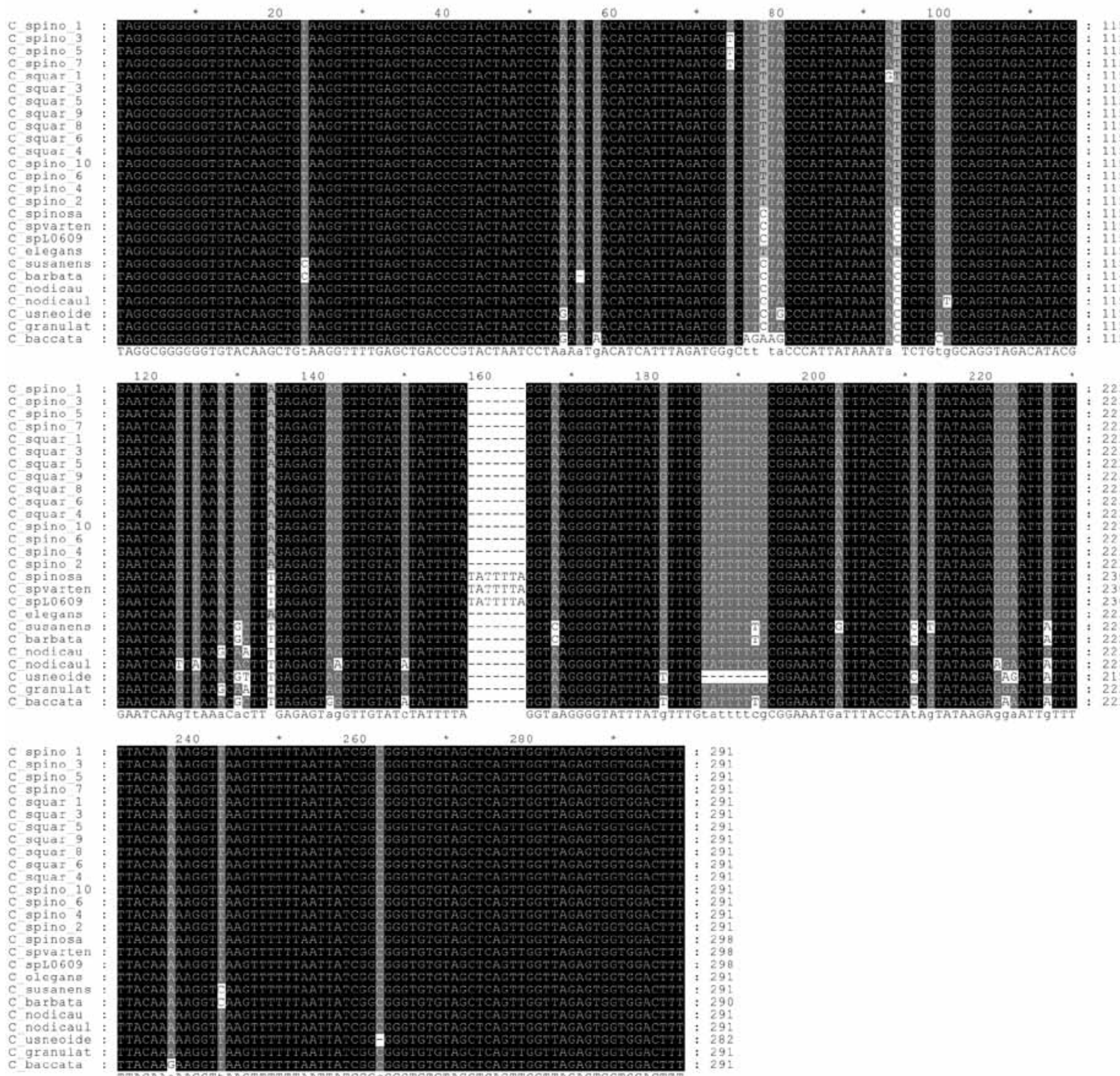

Fig. 3. Sequence alignment for the mt spacer (mt23S-tRNAVal) sequences analyzed in Fig. 2

nally assumed. Interestingly, GIACCONE (1978) had considered that "squarrosa" might be a variety of *C. spinosa* (as *C. spinosa* var. *squarrosa* (De Notarsis) Giaccone) (GUIRY & GUIRY, 2012), but this combination is currently considered as a synonym of *C. squarrosa*. More morphological and molecular studies have to be carried out before a definitive conclusion can be made. And more molecular markers have to be tested to better understand the taxonomy and evolution of the genus *Cystoseira*.

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Molekularna identifikacija smeđe alge C*ystoseira* spp. (Phaeophycae, Fucales) iz Jadranskog mora – preliminarni rezultati

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SAŽETAK

U cilju odabira odgovarajućeg molekularnog biljega koji bi omogućio genetičko razlikovanje među različitim vrstama roda *Cystoseira* u Jadranskom moru, analizirana su DVA molekularna biljega: mitohondrijska 23S rDNA i mitohondrijska razmaknica (mt23S-tRNAVal). U istraživanju su bile korištene dvije vrste *Cystoseira spinosa* i *Cystoseira squarrosa*. Sekvenciranje i usporedba tih fragmenata pokazali su da je regija mt23S rDNA viskoko konzervirana, jer u svih analiziranih jedinki te sekvence nisu pokazivale varijablinost. U strukturi mitohondrijske razmaknice (mt23S-tRNAVal) uočene su dvije mutacije i tri haplotipa; A, B i C. Dok je haplotip B nađen samo u vrste *C. spinosa*, a haplotip C u vrste *C. squarrosa*, najdominatniji haplotip A nađen je kod podjednakog broja jedinki obiju vrsta. U usporedbi s nekoliko odabranih vrsta roda *Cystoseira,* podrijetlom iz drugih dijelova Mediteranskog mora, taj molekularni biljeg mt23S-tRNAVal ipak nije pokazao vrsnu specifičnost. Iako ti preliminarni rezultati pokazuju ograničenu mogućnost mitohondrijske razmaknice (mt23S-tRNAVal) za razlikovanje među jadranskim vrstama roda *Cystoseira*, oni mogu ukazivati i na konspecifičnost istraživanih vrsta odnosno odražavati njihovu relativno nedavnu divergenciju. Daljnja istraživanja će biti potrebna kako bi se poboljšala molekularna identifikacija vrsta ovih smeđih alga.

Ključne riječi: *Cystoseira*, Jadransko more, mitohondrijska DNA, molekularni biljezi, molekularna filogenija