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RESOLVING THE TAXONOMIC STATUS OF THE MYSTERIOUS „GREEN OAK" (*QUERCUS* × *VIRIDIS* TRINAJSTIĆ) FROM CROATIA BASED ON PHYLOGENETIC ANALYSIS

TAKSONOMSKI STATUS MISTERIOZNOGA "ZELENOGA HRASTA" (*QUERCUS* × *VIRIDIS* TRINAJSTIĆ) IZ HRVATSKE, TEMELJEN NA FILOGENETSKOJ ANALIZI

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Summary

The "green oak" is a well-known specific individual oak tree of unknown origin growing near Zadar in Croatia. Depending on the authors, it was described either as a hybrid taxon between *Quercus cerris* L. and *Q. ilex* L. (named *Q.* × *viridis* Trinajstić) or alternatively as a presumed hybrid between *Q. cerris* and *Q. suber* L. To finally resolve the origin of this taxon, we performed molecular analyses and investigated the phylogenetic relationships between the "green oak" and other closely related oak taxa from the surrounding area, including all putative parental species. A total of 16 individuals representing nine *Quercus* L. taxa were investigated based on both plastid (*trn*K-*mat*K and *trn*H-*psb*A) and nuclear (5.8S + ITS2) DNA sequence variation. Placement of the green oak in the phylogenetic relationships between the studied oak taxa does not support *Q. ilex* as one of its parental species but rather indicates that this taxon is in fact *Q. crenata* Lam., reaffirming previous alternative hypothesis that the green oak is a hybrid between *Q. cerris* and *Q. suber.* We therefore confirm the presence of *Q. crenata* in the Croatian flora and based on historical literature survey, we explore and discuss the implication of its occurrence and possible hybridogenic origin in the Croatian territory.

KEY WORDS: Green oak, *Quercus* × *viridis*, *Quercus crenata*, hybrids, phylogeny, taxonomy

INTRODUCTION
UVOD

Oak taxonomy has been an unfailing research topic for decades and continues to be so. This comes as no surprise since oaks (genus *Quercus* L., *Fagaceae*) are among the most important forest trees in the Northern hemisphere from ecological, economic, biodiversity and social points of view. In addition, oaks offer an emblematic example of problematic taxonomy and species status due to extensive hybridization among several taxa (Muir, 2000). Although challenging the classical biological species concept (Burger,

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1975), hybridization in oaks has greatly contributed to their extremely high variation and plasticity; it constitutes an important mechanism of their dispersal and is suggested to play a crucial role in their adaptive potential to changing climate (Rushton, 1993; Petit *et. al.*, 2004; Aitken *et. al.*, 2008).

Our case study focused on resolving the taxonomic status of a specific semi-evergreen oak tree described and known in literature as the "green oak" due to persistence of its thick green leaves deep into the winter (up to end of February). This individual tree grows in the village of Islam Latinski (Zadar County), Northern Dalmatia, Croatia, and has been protected as a nature monument since 1957 (HTRS96 x: 414861.9036, y: 4895526.4636) (Figure 1). It is estimated to be more than 200 years old (Borzan *et. al.*, 1997). To the best of our knowledge, the first literature mention of the "green oak" was by Jedlowski (1955) who presumed that the individual is a probable hybrid between *Q. cerris* L. and *Q. suber* L. Later, Trinajstić (1974a) described this particular individual tree as a hybrid *Quercus* × *viridis* Trinajstić, hybr. nov. suggesting that it is in fact a hybrid probably between *Quercus cerris* f. *austriaca* and *Q. ilex* (Figure 2). He based his description on morphological characters and the fact that *Q. suber* is not growing in the surrounding area. Afterwards, the peculiar green oak has been investigated several times including botanical, biological, taxonomical, detailed morphological as well as wood and leaf anatomy points of view (Siegert, 1995; Vranković and Pernar, 1995; Borzan *et. al.*, 1997; Borzan and Pfeifhofer, 1998; Borzan, 2000; Guttenberger, 2000; Borzan and Stabentheiner, 2002). For example, Borzan (2000) has reported hermaphroditic unseasonal flowering in the green oak which lead to the detailed study of pollen and leaf hairs morphology of this individual compared to the putative ancestors *Q. cerris* and *Q. ilex* by scanning electron microscopy (Borzan and Stabentheiner, 2002). The latter study implied that the green oak does not share any similarities with *Q. ilex* based on these traits. Likewise, Guttenberger (2000) who has also performed measurements of the DNA content of the green oak and related oak species concluded that *Q. ilex* does not seem to be one of the parental species of the green oak.

Finally, the latest review on the origin of this particular oak by Muller *et. al.* (2003) suggested that this individual shares much more similarities with *Q. cerris* compared to *Q. ilex*; the authors conclude that all gathered studies of this taxon support the opinion given by Jedlovski (1955) and not the description by Trinajstić (1974a). However, the origin of this intriguing taxon remains still uncertain to date, and all

Figure 1. The „Green oak" (*Quercus* × *viridis* Trinajstić) individual growing in Islam Latinski near Zadar, Croatia.
Slika 1. *Stablo „zelenoga hrasta"* (Quercus × viridis *Trinajstić*), *Islam Latinski pored Zadra u Hrvatskoj.*

Figure 2. Herbarium specimen of the „Green oak" individual (*Quercus* × *viridis* Trinajstić) from Ravni Kotari collected by prof. emer. Ivo Trinajstić in 1965. (The specimen is deposited in the Herbarium collection of The Croatian Natural History Museum (CNHM) under inventory number 600:ZAG;3297:BOT).

Slika 2. Herbarijski primjerak „zelenoga hrasta" (Quercus × viridis *Trinajstić*) *s lokaliteta Ravni Kotari kojega je sakupio prof. emer. Ivo Trinajstić 1965. godine. (Primjerak je pohranjen u Herbarijskoj zbirci Hrvatskog prirodoslovnog muzeja (CNHM) pod inventarnom oznakom 600:ZAG;3297:BOT).*

cited authors recommended the use of molecular data and DNA analysis to definitely assess its taxonomic status.

In this study, we carried out for the first time a phylogenetic analysis of the "green oak" in comparison to other closely related oak species from the surrounding region, including all putative parental species: *Q. cerris*, *Q. ilex* and *Q. suber*. A further oak tree from Croatia displaying similar morphology to the "green oak" individual, as well as members of *Q. crenata* Lam., i.e. the supposed stable hybrid between *Q. cerris* and *Q. suber* (Bellarosa *et. al.*, 2005; Cristofolini *et al.*, 2005; Conte *et. al.*, 2007) from Italy and Slovenia, were included in the dataset. To finally resolve the origin of the green oak tree, we combined both plastid and nuclear DNA markers widely used for DNA barcoding in plants and which have proven to be helpful in taxonomic identification and delimitation of some closely related Euro-Mediterranean oak taxa (Simeone *et. al.*, 2013).

MATERIALS AND METHODS
MATERIJAL I METODE

Plant material – *Biljni materijal*

We collected leaf samples from the "green oak" individual (*Q.* × *viridis*) growing in the village of Islam Latinski near Zadar, as well as from another putative green oak growing near Rijeka (Donje Jelenje) where unusual evergreen oak individuals of unknown origin have been observed (J. Franjić, personal observation) (Table 1). In addition, leaf samples from 1-3 individuals were collected from all closely related oak taxa (*Q. cerris, Q. suber, Q. ilex*, *Q. coccifera* L. and *Q. crenata*) from Croatia, Slovenia, Italy and Greece to maximally cover the geographic area surrounding the target "green oak". Finally, samples from single individuals of three more distant oak species belonging to the infrageneric group *Quercus* (*Q. robur* L., *Q. petraea* /Matt./ Liebl. and *Q. pubescens* Willd.) were also investigated and used as outgroups. In total, nine *Quercus* taxa were included in the analyses (Table 1).

DNA extraction and amplification – *Izolacija i umnažanje DNK*

DNA extractions from silica-gel dried leaves were performed with the DNeasy Plant Minikit (QIAGEN), following the manufacturer's instructions. In a search for maximum variability in our taxon-based study, we used two highly variable plastid regions (*trn*K-*mat*K and *trn*H-*psb*A), and the nuclear ribosomal region 5.8S + ITS2 (Simeone *et al.*, 2013). Plastid primers were the same as in Piredda *et. al.* (2011); ribosomal oligonucleotides were designed as follows: 5'-ACG ACT CTC GGC AAC GGA TA-3' (5.8S_Fw), 5'-CAG CGG GTA GTC CCG CCT GA-3' (25S_Rev). DNAs (ca. 40 ng) were amplified with RTG PCR beads (GE Healthcare) in 25 µl final volume according to the manufacturer's protocol. Thermocycling conditions were: 94° for 3 min, followed by 35 cycles of 94° for 30 sec, 53° for 40 sec and 72° for 40 sec, with a final extension step of 10 min at 72° (plastid loci), and 98° for 3 min, followed by 35 cycles of 98° for 30 sec, 60° for 30 sec and 72° for 30 sec, with a final extension step of 5 min at 72° (ITS region). PCR products were cleaned with Illustra DNA and Gel Band Purification Kit (GE Healthcare), and eluted in 30 µl type 6 elution buffer. Standardized aliquots were then submitted to Eurofins MWG Operon (https://www.eurofinsgenomics.eu) for sequencing. Electropherograms were edited with CHROMAS 2.3 (www.technelysium.com.au) and checked visually.

Phylogenetic analyses – *Filogenetske analize*

All sequences were first aligned with MUSCLE (Edgar, 2004) and checked by eye. We calculated summary statistics of the aligned sequences (number of variable sites – V,

Table 1. *Quercus taxa* and sampled plant material in the present study. Voucher and GenBank accession numbers of sequences (https://www.ncbi.nlm.nih.gov/genbank/) are provided for each sample. Division to infrageneric groups according to Denk and Grimm (2010) and Hubert et al. (2014).

Tablica 1. Taksoni roda Quercus i uzorkovane jedinke zastupljene u ovom istraživanju. Za svaki uzorak navedeni su brojevi vaučera i pristupni brojevi sekvenci pohranjenih u banci gena GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Podjela na skupine prema Denk i Grimm (2010) i Hubert i sur. (2014).

Taxa *Takson*	Number of samples *Broj jedinki*	Infrageneric group *Skupina*	Geographic origin *Podrijetlo jedinki*	Sampling site *Lokalitet*	Voucher* *Vaučer**	*trn*H-*psb*A	*trn*K-*mat*K	5.8S + ITS2
Q. × *viridis* Trinajstić 1	1	-	Croatia	Rijeka, Donje Jelenje	041x054.01/HR	HE591340	HE611289	HE591369
Q. × *viridis* Trinajstić 2	1	-	Croatia	Zadar, Islam Latinski	041x054.02/HR	HE591341	HE611288	HE591370
Q. coccifera L.	1	Ilex	Croatia	Dubrovnik	042.01/HR	HE591300	HE583657	HE583749
Q. ilex L.	1	Ilex	Croatia	Pula	046.01/HR	HE591302	HE583659	HE583751
Q. cerris L.	1	Cerris	Croatia	Učka	041.01/HR	HE591292	HE583649	HE583741
Q. cerris L.	1	Cerris	Italy	Stigliano	041.01/IT.MT	HE591248	HE583605	HE583673
Q. cerris L.	1	Cerris	Greece	Elafina	046.01/GR	HE591291	HE583648	HE583740
Q. crenata Lam.	1	Cerris	Italy	Latium, Tuscania	041x054/IT.VT	HE591250	HE583607	HE583675
Q. crenata Lam.	1	Cerris	Italy	Trentino, Condino	041x054/IT.TN	HE591249	HE583606	HE583674
Q. crenata Lam.	1	Cerris	Slovenia	Makole	041x054/SL	HE591293	HE583650	HE583742
Q. suber L.	1	Cerris	Croatia	Pula	054.01/HR	**	**	**
Q. suber L.	1	Cerris	Italy	Latium, Tuscania	054.01/IT.VT	HE591254	HE583611	HE583679
Q. suber L.	1	Cerris	Italy	Apulia, Bari	054.01/IT.BA	HE591256	HE583613	HE583681
Q. petraea (Matt.) Liebl.	1	Quercus	Italy	Latium, Viterbo	048.01/IT.VT	HE591284	HE583641	HE583709
Q. pubescens Willd.	1	Quercus	Italy	Latium, Viterbo	049.01/IT.VT	HE591288	HE583645	HE583713
Q. robur L.	1	Quercus	Italy	Latium, Rome	051.01/IT.RM	HE591286	HE583643	HE583711
Total *Ukupno*	16							

* Vouchers with institutional accession numbers are stored at the Forest DNA Bank (MFDB) of the University of Tuscia, Viterbo and available upon request.

** Vaučeri sa pristupnim brojevima pohranjeni su u Forest DNA Bank (MFDB) na Sveučilištu Tuscia u Viterbu i dostupni su na zahtjev.*

** Identical to HE591254, HE583611 and HE583679, see text. *** Identično kao HE591254, HE583611 i HE583679, vidi tekst*

number of Parsimony informative sites – Pi, number of haplotypes – h, and nucleotide diversity - π) using Arlequin v. 3.5.2.2 (Excoffier and Lischer, 2010). The phylogenetic analyses were performed separately on the combined plastid DNA dataset (*trn*K-*mat*K + *trn*H-*psb*A sequences) and on the nuclear dataset (5.8S + ITS2). We first constructed a plastid haplotype and a nuclear ribotype networks, based on the median-joining network algorithm (Bandelt *et. al.*, 1999) as implemented in the PopArt software (Leigh and Bryant, 2015). Next, to assess the phylogenetic relationships among the studied oak taxa based on the nuclear sequence dataset, we constructed a phylogenetic tree using a maximum parsimony (MP) method with bootstrap analyses of 1000 replicates to test for the branch support. The analysis was carried out using the MEGA v. 7.0.26 and Seaview v4.6.2 software (Gouy *et. al.*, 2010; Tamura *et al.* 2016) with default parameters (gaps were treated as unknown states). Finally, because the position of hybrid species in a classic phylogenetic tree is often doubtful, we also computed a rooted consensus cluster network based on nuclear sequences to identify putative hybridization events leading to the formation of the "green oak". This network was based on a set of 1000 original trees obtained with a MP analysis and with a 20 % threshold (minimum support) for a cluster to be included in the procedure using Dendroscope v. 3.5.9 (Huson and Scornavacca, 2012)

RESULTS
REZULTATI

Our dataset included 16 sequences of both plastid and nuclear regions for nine oak taxa. Alignment of the *trn*H-*psb*A region had 538 characters (bp) in length, 22 variable sites and 7 parsimony informative sites. Alignment of the *trn*K-*mat*K region displayed 694 characters, 14 variable sites and 12 parsimony informative sites, while the alignment of the combined plastid DNA dataset resulted in a total length of 1232 sites, 36 of which were variable and 19 parsimony informative (Table 2). The alignment of the nuclear dataset resulted in the total length of 371 sites of which 21 were variable and 13 parsimony informative (Table 2).

In total, five haplotypes were identified based on the combined plastid sequences with a low nucleotide diversity π = 0.0073 (Table 2). The plastid DNA haplotype network showed that one haplotype was shared among 11 studied individuals representative of four oak taxa, namely *Q. cer-*

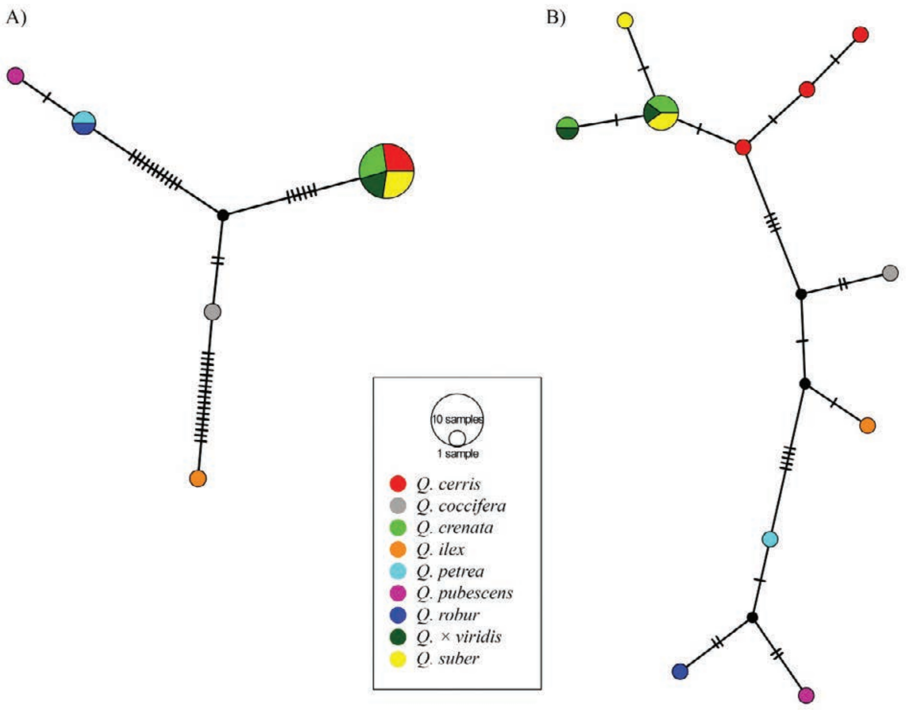

Figure 3. Median-joining haplotype/ribotype network of nine *Quercus taxa* based on the a) plastid (trnK-matK + trnH-psbA) sequences b) nuclear (5.8S + ITS2) sequences. Each circle depicts a distinct haplotype/ribotype. Colours indicate different *Quercus taxa* and circle sizes are proportional to the haplotype/ribotype frequency in the data set. Hatch marks correspond to the number of mutational steps between haplotypes/ribotypes and black circles depict haplotypes/ribotypes not sampled. Networks were created using PopART v.1.7 software (http://popart.otago.ac.nz).

Slika 3. Mreža haplotipova/ribotipova dobivena „median joining“ metodom koja prikazuje odnose devet taksona roda Quercus temeljem a) kloroplastnih (trnK-matK i trnH-psbA) i b) jezgrinih (5.8S + ITS2) DNK sekvenci. Svaki krug predstavlja pojedini haplotip/ribotip. Boja kruga označava različite taksone roda Quercus a veličina kruga je proporcionalna frekvenciji pojedinog haplotipa/ribotipa. Okomite crtice predstavljaju broj mutacijskih koraka između haplotipova/ribotipova, dok crni kružići označavaju haplotipove/ribotipove koji nisu zastupljeni, tj. uzorkovani u ovom istraživanju. Filogenetska mreža dobivena je pomoću programa PopART v.1.7 (http://popart.otago.ac.nz).

ris, Q. suber, Q. crenata and *Q.* × *viridis* (Figure 3a). Another plastid haplotype was shared between *Q. robur* and *Q. petraea* individuals while *Q. ilex*, *Q. coccifera* and *Q. pubescens* displayed different, unique haplotypes. The haplotype network showed that the five haplotypes are arranged according to three lineages, each representing the three main infrageneric groups encompassing the here studied oaks: Group *Cerris* (*Q. cerris*, *Q. suber*, *Q. crenata*), Group *Ilex* (*Q. ilex* and *Q. coccifera*) and Group *Quercus* (*Q. robur*, *Q. petraea* and *Q. pubescens*). Interestingly, *Q.* × *viridis* individuals were all included in the only haplotype of the *Cerris* group, diverging from the *Q. ilex* haplotype by 27 mutational steps (Figure 3a).

The nuclear dataset was characterized by 11 ITS2 + 5.8S ribotypes (Figure 3b) and higher nucleotide diversity π = 0.01617 (Table 2). In contrast to the obtained plastid haplotypes, the majority of the included *Quercus* taxa as well as some individuals of the same species showed private ITS2 + 5.8S sequence variants. In total, nine ribotypes occurring in single individuals were detected. Two variants were shared among different taxa: one between *Q. suber, Q. crenata* and *Q.* × *viridis* (from Rijeka) and the other between *Q. crenata* and *Q.* × *viridis* (from Zadar) (Figure 3b). Three ribotypes were detected in *Q. cerris* and two in *Q. suber* and in *Q.* × *viridis.* Similar to the cpDNA network, lineages corresponding to the three infrageneric groups could be recognized. Ribotypes found in the *Q.* × *viridis* individuals were included in the *Cerris* lineage, separated from the ribotype displayed by *Q. ilex* by at least 10 mutational steps.

The reconstructed MP phylogenetic relationships of the nuclear ribosomal sequences revealed three main clades, still corresponding to the three *Quercus* infrageneric groups (Figure 4). Within the *Cerris* clade, the putative *Q.* × *viridis* individual from Islam Latinski (Zadar) and the other putative *Q.* × *viridis* individual from Donje Jelenje (Rijeka), formed a subclade together with *Q. suber* and *Q. crenata* individuals from Croatia, Slovenia and Italy, with no or very low resolution, and separated from the *Q. cerris* sequences. In particular, *Q.* × *viridis* individual from Zadar (Croatia) was placed as a sister taxon next to the sampled *Q. crenata* from the Latium region in Italy (Figure 4). Separation of the *Q.* × *viridis* individuals from those belonging to the

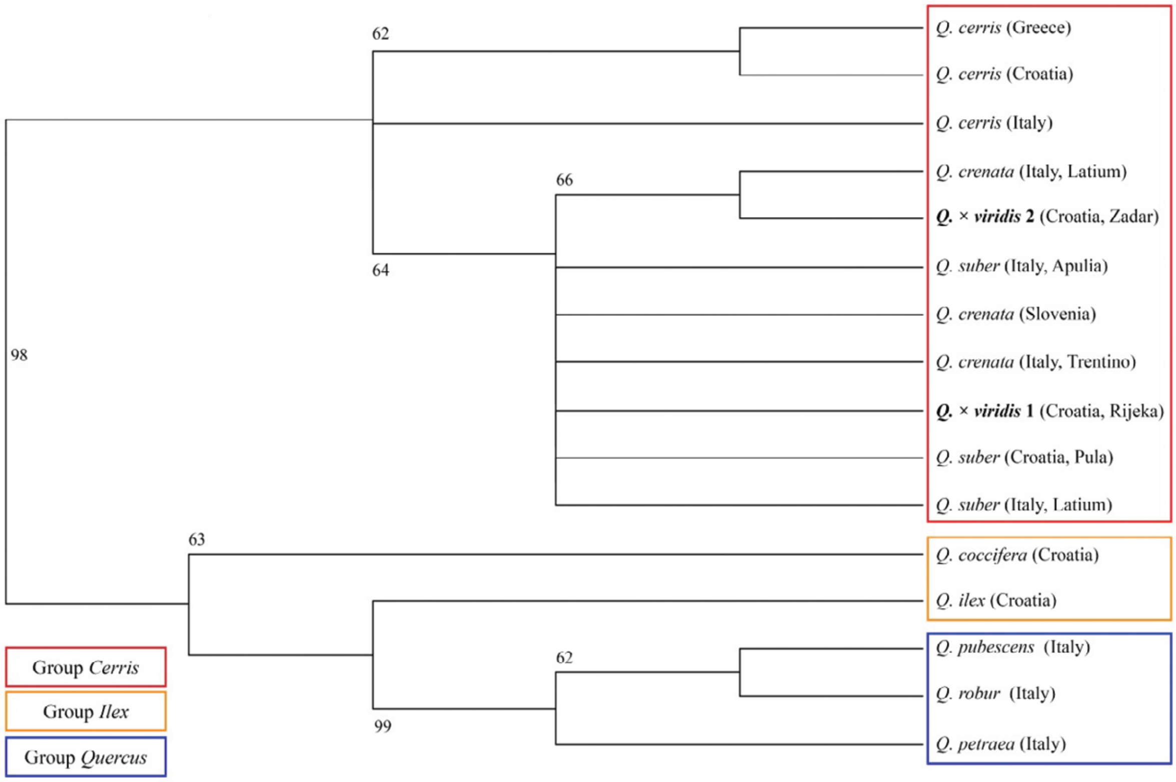

Figure 4. Maximum-parsimony 100 % consensus tree based on nuclear sequences (5.8S +ITS2 region) of nine *Quercus taxa*, including the two putative green oak individuals (*Q.* × *viridis*) indicated in bold. Origin of individuals is indicated in brackets. Bootstrap values (>50 %) based on 1000 replications are shown above the branches/at branch nodes.

Slika 4. Strogo dogovorno stablo (100 %) konstruirano metodom makimalne štedljivosti na temelju jezgrinih sekvenci (5.8S +ITS2 regija) devet taksona iz roda Quercus. Položaj jedinki taksona Q. × viridis *prikazan je podebljano. Podrijetlo jedinki naznačeno je u zagradama. Podrška grananja unutar filogenetskoga stabla u obliku „Bootstrap" vrijednosti (>50 %) temeljem 1000 ponavljanja prikazana je na čvorovima grana.*

members of the *Ilex* infrageneric group, *Q. ilex* and *Q. coccifera*, was evident. Finally, the rooted consensus cluster network clearly indicated close relationships between all members of the *Cerris* subclade, and further suggested the hybrid origin of the *Q.* × *viridis* with *Q. suber* as one of the parental species (Figure 5).

DISCUSSION
RASPRAVA

Our work represents the first attempt to resolve the taxonomic status of the "green oak" (*Q.* × *viridis*), described by Trinajstić (1974a) as a putative hybrid between *Q. cerris* and *Q. ilex*, based on molecular data. We used both plastid and nuclear DNA sequences to carry out a phylogenetic analysis involving a total of nine oak taxa from a wide geographic range surrounding the investigated oak, including all possible parental species. All our results of phylogenetic relationships among the studied oak taxa do not support *Q. ilex* as one of the parental species of the green oak. Rather, they confirm that the investigated green oak individuals are actually *Q. crenata* Lam., a presumed hybrid between *Q. cerris* and *Q. suber*, as first suggested by Jedlowski (1955). However, the two putative *Q.* × *viridis* individuals included in this study did not show identical phylogenetic pattern.

Both putative *Q.* × *viridis* individuals shared the same cpDNA haplotype (Figure 3) which was also present in all other cerroid oaks included in this study (*Q. cerris, Q. suber* and *Q. crenata*). This is however not surprising as the used cpDNA barcoding regions (*trn*K-*mat*K and *trn*H-*psb*A) were shown to have low differentiation rates below the infrageneric level and thus relatively low efficiency to discriminate between closely related *Quercus* taxa, particularly for the Group *Cerris* (Simeone *et. al.*, 2013). Interspecific sharing of plastid haplotypes is also well documented for the white oaks of the infrageneric groups *Quercus* and *Ilex* across wide geographic areas (Petit *et. al.*, 2002; Slade *et. al.*, 2007; Vitelli *et al.*, 2017).

On the other hand, the nuclear 5.8S + ITS2 region has shown to have a better discriminatory power in oak phylogeny compared to the used plastid regions (Bellarosa *et. al.*, 2005). In this study, the two *Q.* × *viridis* individuals displayed slightly different ribotypes, separated by only one mutational step (Figure 3). In addition, the "famous" green

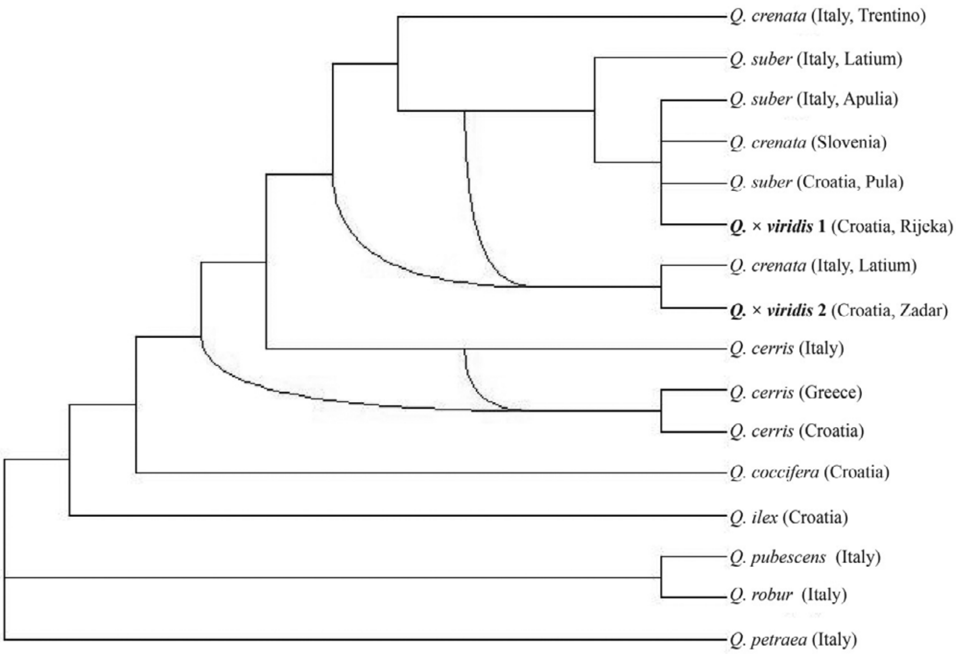

Figure 5. Rooted consensus network based on nuclear sequences (5.8S + ITS2 region) computed using Dendroscope from a set of 1000 of trees obtained with a Maximum Parsimony analysis. The resulting network represents clusters occurring, at least, in the 20 % of all trees in their full resolution. Putative hybridization events are represented by blue lines.

Slika 5. Ukorijenjena dogovorna mreža temeljem jezgrinih sekvenci (5.8S + ITS2 regija) dobivena u programu Dendroscope od 1000 originalnih stabala maksimalne štedljivosti. Prikazana filogenetska mreža predstavlja klastere koji se pojavljuju u barem 20 % svih ulaznih stabala. Potencijalni procesi/putevi hibridizacije prikazani su plavim linijama.

oak individual near Zadar shared its ribotype with *Q. crenata* from Italy (Latium), while the putative green oak individual from Rijeka exhibited the same ribotype as *Q. suber* from Croatia (Pula) and Italy (Latium) as well as *Q. crenata* from Slovenia and Italy (Trentino). Likewise, placement of both *Q.* × *viridis* from Zadar and Rijeka as sister taxa to *Q. crenata* and *Q. suber* in the MP tree indicates that these taxa (*Q. suber*, *Q. crenata* and *Q.* × *viridis*) are indeed highly related at the nuclear level. Borzan (2002) already pointed out that "a visit to the Herbarium Patavinum, Padua (Italy), and to the Erbario Centrale Italiano »Filippo Parlatore«, Sezione di Botanica, Museo di Scienze Naturali, Firenze (Italy), revealed that the herbarium samples of the "green oak" from Zadar are very similar to those kept in these Italian botanical institutions under the name *Q. crenata* and *Q. pseudosuber* Santi". Individuals assigned to the complex *Q. crenata* – *Q. pseudosuber* are in fact traditionally acknowledged as stabilised hybrids between *Q. cerris* × *Q. suber* and the latest paper on this topic concluded that *Q. crenata* Lam. is the correct name for this nothospecies (Cristofolini and Crema 2005; Cristofolini *et. al.*, 2017). This oak taxon (the "false cork oak") is known to occur in southern France and Italy where the two supposed parental species are found in sympatry; however, scattered *Q. crenata* individuals can be also found in Northeastern Italy, western Slovenia and Croatia, where *Q. suber* is currently out of its distribution range (Cristofolini and Crema 2005; Bellarosa *et. al.*, 2005). Thus, the origin of these *Q. crenata* individuals is still under debate (Cristofolini and Crema, 2005; Conte *et. al.*, 2006; Schirone *et. al.*, 2015).

Table 2. Summary statistics of the aligned sequences.

Tablica 2. Sumarna statistika sravnjenih sekvenci.

	trnH-psbA	*trnK-matK*	cpDNA	5.8S + ITS2
N	16	16	16	16
Length (bp) *Dužina (pb)*	538	694	1232	371
V	22 (4.3 %)	14 (2 %)	36 (3 %)	21 (5.7 %)
Pi	7 (1.4 %)	12 (1.7 %)	19 (1.6 %)	13 (3.5 %)
π	0.0082	0.0067	0.0073	0.0162

N – number of sequences, V – number of variable sites, Pi – number of Parsimony informative sites, π – nucleotide diversity.

N – broj sekvenci, V – broj varijabilnih mjesta, Pi – broj informativnih mjesta, π – nukleotidna raznolikost.

For example, some authors included Croatia in the natural range of *Q. suber* (Muller, 1900; see references in Schirone *et. al.*, 2015 and Euro+Med PlantBase) because this oak species has been well documented in older floristic literature from several localities across Croatia. These include Istria and particularly the Šijana forest, near Pula, where the largest and oldest known cork oak stand in Croatia occurs (Trinajstić, 2006 and references therein). In addition, isolated *Q. suber* trees are known from several localities in Dalmatia: Dundo forest on the island of Rab, on Mljet and in Blato on the island of Korčula (Hirc, 1903-1912; Trinajstić, 2006). Moreover, individuals of *Q. pseudosuber* (= *Q. crenata*) were historically reported from many sites across Istria and the Kvarner region (Šijana forest, Strobl, 1872; Pericin 2001, Veruda forest, Freyn, 1877; Dragonja, Tinjan, Pazin, Mirna river, Lovran and Volosko, Ascherson and Graebner, 1908-1913; Pula and Volosko, Schneider, 1906; Cres, Hirc, 1916; Kvarner, Hayek, 1924; Cres and Rab island, kanjon Rječine, Lovrić, 1981), as well as from Dalmatia (Richter, 1897; Adamović, 1911; Hayek, 1924, 1927). Later, Trinajstić (2006) suggested that in fact all *Q. pseudosuber* individuals reported from Croatia belong to *Q. suber* and concluded that the taxon *Q. crenata* is not present in Croatian flora. However, our results provide clear evidence that *Q. crenata* individuals do occur in Croatia and confirm similar findings of previous studies (Lovrić, 1981; Cristofolini and Crema, 2005; Perinčić, 2010; Simeone *et. al.*, 2009, 2013; Schirone *et. al.*, 2015). For example, more recently *Q. crenata* was recorded in Zadar, nevertheless it was cultivated in the Vladimir Nazor Park which dates from the end of the 19th century (Perinčić, 2010; Nikolić 2017). Thus, it remains an open question whether this taxon could have been established in Croatia naturally or has been man-mediated. Based on similar findings of *Q. suber* and *Q. crenata* individuals on the eastern side of the Adriatic sea, several authors hypothesized that *Q. suber* first originated in the Eastern Mediterranean (probably surviving in the Balkan Peninsula), expanded westward and finally got extinct in the East Mediterranean (Bellarosa *et. al.*, 2005; Simeone *et. al.*, 2009; Schirone *et. al.*, 2015). Under this hypothesis, *Q. suber* and *Q. cerris* would have been naturally co-occurring until a recent past, possibly giving rise to occasional hybridizations. However, up to date this scenario has not been generally accepted due to lack of strong scientific evidence and is a subject of ongoing debate (c.f. Magri *et al.*, 2007).

The two "green oak" individuals included in this study did not show identical genetic patterns, indicating a different descent. If we assume an hybridogenic origin, the phylogenetic position of *Q.* × *viridis* from Rijeka, clustering among *Q. crenata* individuals from Slovenia and Trentino (Northern Italy) and *Q. suber* (Figure 3, Figure 4) suggest that, at least for the region of Istra and Kvarner, it is plausible to infer a natural origin of this tree, since both parental species (*Q. cerris* and *Q. suber*) grow in this area since, at least, four generations (Trinajstić, 2006). On the other hand, it is highly unlikely that the "green oak" from Islam Latinski (Zadar) is a natural hybrid of the two parental species, since *Q. suber* is absent from the surrounding area and the nearest known cork oak trees grow on the island of Rab (cca 90 km away), where they were planted at the beginning of the 20th century (Šurić, 1933), thus after the "green oak" individual was already established. Placement of this green oak individual in the phylogenetic relationships with *Q. crenata* from Latium in Italy indicates a possibility that this particular oak originated from this region where *Q. crenata* is particularly widespread. It was likely planted in the village of Islam Latinski a long time ago since long distance pollen dispersal from *Q. crenata* and /or *Q. suber* from Central Italy does not seem highly probable. However, extinction/progressive rarefaction of its wild, close-by relatives that were part of a common, little differentiated gene pool is also probable, and more extensive samplings would be clearly needed to accurately establish autochthony or the provenance origin of this important tree.

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Sažetak

„Zeleni hrast" je već dugo poznato i specifično stablo hrasta nepoznatoga podrijetla, koje raste u selu Islam Latinski blizu Zadra u Hrvatskoj, a procjenjuje se da je staro preko 200 godina (Slika 1). Svoje ime zahvaljuje činjenici što njegovo debelo kožasto zeleno lišće ostaje na stablu duboko u zimu. Zeleni hrast prema našim saznanjima po prvi puta u literaturi spominje Jedlowski (1955) te pretpostavlja kako se radi o križancu cera (*Quercus cerris* L.) i hrasta plutnjaka (*Q. suber* L.). Kasnije ga Trinajstić (1974) opisuje kao novi takson *Quercus* × *viridis* Trinajstić, hybr. nov. koji je prema njegovom mišljenju križanac cera (*Q. cerris*) i hrasta crnike (*Q. ilex* L.) (Slika 2). Nakon toga ovaj hrast bio je predmet mnogih istraživanja, kako taksonomskih, tako i ekoloških, botaničkih, anatomskih i morfoloških. Posljednji pregledni članak o zelenom hrastu (Müller i sur., 2003) zaključuje kako rezultati većine dosadašnjih istraživanja podupiru mišljenje koje je dao Jedlowski, a ne opis taksona od Trinajstića. Međutim, do danas taksonomski status zelenoga hrasta i njegovo podrijetlo ostaju neizvjesni. Stoga je u ovome radu po prvi puta provedena analiza filogenetskih odnosa zelenoga hrasta na temelju molekularnih biljega u odnosu na ostale blisko srodne vrste hrasta koje rastu u njegovoj široj regiji, uključujući sve potencijalne roditeljske vrste (*Q. cerris, Q. ilex, Q. suber* i *Q. coccifera* L.) kao i takson *Q. crenata* Lam. koji je poznat kao stabilni križanac vrsta *Q. cerris* i *Q. suber* u Italiji. Također su u analizu uključeni predstavnici skupine *Quercus* (*Q. robur* L., *Q. petraea* /Matt./ Liebl. i *Q. pubescens* Willd.) kao vanjske grupe. Ukupno naš set podataka uključivao je devet taksona i 16 jedinki roda *Quercus* L. (Tablica 1). Kako bismo konačno utvrdili taksonomski status zelenoga hrasta koristili smo kloroplastne (*trn*K-*mat*K i *trn*H-*psb*A) i jezgrine (5.8S + ITS2) DNK biljege (tzv. DNK barkod regije) na temelju kojih smo rekonstruirali srodstvene odnose pomoću mreže haplotipova/ribotipova (Slika 3) te pomoću filogenetskoga stabla dobivenoga metodom maksimalne štedljivosti (Slika 4 i 5). Rezultati filogenetskih odnosa između zelenoga hrasta i ostalih zastupljenih taksona u ovome istraživanju (Slika 3, 4 i 5) ne podržavaju teoriju da je *Q. ilex* jedna od njegovih roditeljskih vrsta. Umjesto toga, molekularna filogenija nedvojbeno dokazuje kako je zeleni hrast zapravo takson poznat pod prihvaćenim nazivom *Q. crenata,* te potvrđuje alternativnu hipotezu kako se radi o križancu između vrsta *Q. cerris* i *Q. suber*. Pojedini autori u starijoj literaturi već su navodili svojtu *Q. crenata na* temelju morfoloških karakteristika za područje Istre i Kvarnera te Dalmacije, koristeći se uglavnom sinonimom *Q. pseudosuber* Santi (Strobl, 1872; Freyn, 1877; Richter, 1897; Schneider, 1906; Ascherson i Graebner, 1908-1913; Adamović, 1911; Hirc, 1916; Hayek, 1924, 1927; Lovrić, 1981). Međutim, kasnije Trinajstić (2006) ipak zaključuje kako takson *Q. crenata* nije zastupljen u hrvatskoj flori te je do danas ovo pitanje ostalo dvojbeno. U novije vrijeme vrsta *Q. crenata* zabilježena je samo u kulturi u Perivoju Vladimira Nazora u Zadru (Perinčić, 2010; Nikolić 2017). Na osnovi naših rezultata temeljnih na molekularnoj filogeniji sa sigurnošću zaključujemo da je vrsta *Q. crenata* prisutna u hrvatskoj flori.

KLJUČNE RIJEČI: zeleni hrast, *Quercus* × *viridis*, *Quercus crenata*, hibridi, filogenija, taksonomija