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Zaslužuje li *Sibirea croatica* (Degen) status vrste?

Does *Sibirea croatica* (Degen) deserve species status?

Dalibor Ballian[a,b,c,*], Tine Grebenc[b], Gregor Božič[b], Hojka Kraigher[b]

[a]Univerzitet u Sarajevu, Šumarski fakultet / University of Sarajevo, Faculty of Forestry, Zagrebačka 20, 71000 Sarajevo, Bosna i Hercegovina /Bosnia and Herzegovina

[b]Gozdarski inštitut Slovenije / Forestry Institute of Slovenia Večna pot 2, 1000 Ljubljana, Slovenija / Slovenia

[c]Akademija nauka i umjetnosti Bosne i Hercegovine / Academy of Sciences and Arts of Bosnia and Herzegovina; Bistrik 7, 71000 Sarajevo, Bosna i Hercegovina / Bosnia and Herzegovina

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SAŽETAK

U ovom je radu hrvatska sibireja (*Sibiraea croatica* Degen), rijetka i endemična vrsta hrvatske i bosanskohercegovačke flore, kao njihov tercijarni relikt, uspoređena s altajskom sibirejom (*S. altaiensis* (Laxm.) C. K. Schneider). Svi uzorci za istraživanje sibireja prikupljeni su s četiri lokacije, jedne u Bosni i Hercegovini, jedne u Hrvatskoj i dvije u Rusiji. Analizirane su njihova genetička struktura, genetička raznolikost i genetička diferencijacija populacije, njihove genetičke karakteristike i odnos populacije *Sibiraea croatica* Deg., koja raste na području Hrvatske i Bosne i Hercegovine, te kako se razliku-

SUMMARY

In this paper, the Croatian sibirea (*Sibiraea croatica* Degen), a rare and endemic species of the Croatian and Bosnian-Herzegovinian flora, as well as their tertiary relict, was compared with Altaic sibirea (*S. altaiensis* (Laxm.) C. K. Schneider). For the research, all samples of sibirea were collected from four locations, one in Bosnia and Herzegovina, one in Croatia and two in Russia. Their genetic structure, genetic diversity, genetic differentiation of the population and their genetic characteristics were analyzed as well as the relationship of the population of *Sibiraea croatica* Deg. which grows in the regions of Croatia and Bosnia-Herzegovina as opposed to Altaic sibirea (*S. altaiensis* (Laxm.)

* Adresa autora za dopisivanje / *Author's correspondence address*:
E-pošta / *E-mail*: balliandalibor9@gmail.com

je od altajske sibireje (*S. altaiensis* (Laxm.) C. K. Schneider), iz južne Rusije (umjetni nasad) i južnog Sibira. U analizi DNA korišteno je 16 početnica koje su kombinirane tijekom amplifikacije. Na temelju provedenih molekularnih analiza regija u genomskoj i kloroplastnoj DNA utvrđena je njihova genetska bliskost. Stoga se predlaže da hrvatska sibireja (*Sibiraea croatica* Deg.) nije zasebna vrsta, već bi se trebala prepoznati na nižem taksonomskom rangu kao ekotip altajske sibireje (*S. altaiensis* (Laxm.) C. K. Schneider). Temeljem filogenetske analize ITS regija u genomskoj rDNA filogenetski položaj *Sibiraea* spp. potvrđen je unutar familije *Rosaceae*.

KLJUČNE RIJEČI: hrvatska sibireja (*Sibiraea croatica*), molekularne analize, populacija

C. K. Schneider), from Southern Russia (plantation) and Southern Siberia. During the DNA analysis, 16 primers were used, which were combined at amplification. On the basis of molecular analyses of regions in genomic and chloroplast DNA, their genetic closeness was determined. Therefore, the authors suggest that the Croatian sibirea (*Sibiraea croatica* Deg.) is not a separate species, but should be recognized at a lower taxonomic rank as the ecotype of the Altaic sibirea (*S. altaiensis* (Laxm.) C.K. Schneider). Based on the phylogenetic analysis of internal transcribed spacer (ITS) regions in genomic ribosomal genes (rDNA), the phylogenetic position of *Sibiraea* spp. was confirmed within the *Rosaceae* family.

KEYWORDS: Croatian sibirea (*Sibiraea croatica* Deg.), molecular analyses, population

UVOD

Davne 1997. autor (D. Ballian, *op. ur.*) se počeo zanimati za jednu sitnu grmoliku endemičnu biljku poznatu kao hrvatska sibireja (*Sibiraea altaiensis* (Laxm.) C. K. Schneid. subsp. *croatica* Degen. Syn: *S. laevigata*), koja u Bosni i Hercegovini raste na samo nekoliko mjesta. Nalazi se na planini Čvrsnici i Čabulji, na sjeveru Hercegovine (slika 1). Osim što raste u Bosni i Hercegovini kao rijetka i endemična vrsta, nalazi se i u hrvatskoj flori. Hrvatska sibireja prema mnogim autorima [1-4] ima vrlo malo i disjunktno područje rasprostranjenosti na zapadnom Balkanu. Uglavnom raste na vrlo nepristupačnim kamenitim padinama Dinarida, između 800 i 1 500 m nadmorske visine.

U Hrvatskoj raste na području sjevernog i srednjeg Velebita, po rubovima šu-

INTRODUCTION

Back in 1997, the author (D. Ballian, *Ed. note)* became interested in a small bushy endemic plant known as Croatian sibirea (*Sibiraea altaiensis* (Laxm.) C.K. Schneid. subsp. *croatica* Degen. Syn: *S. laevigata*), which in Bosnia and Herzegovina grows in only a few places. It is located on the mountain Čvrsnica and Čabulja, in the north of Herzegovina (*Figure 1*). Although it grows in Bosnia and Herzegovina as a rare and endemic species, it is also found in the Croatian flora. According to many authors [1-4], Croatian sibirea has a very small and disjunct distribution area in the Western Balkans. It mainly grows on the very inaccessible rocky slopes of the Dinarides, between 800 and 1 500 m above sea level.

In Croatia, it grows in the area of northern and central Velebit, along the edges of black hornbeam

ma i šikara crnoga graba, te primorskih šuma obične bukve [2]. Zbog depopulacije i izostanka ljudske djelatnosti otvorena staništa zarastaju, pa grmovi nestaju prirodnom sukcesijom vegetacije. Slično je i u Bosni i Hercegovini gdje joj je areal ograničen na planine Čvrsnicu i Čabulju. Tu raste na vapnenačkim podlogama, otvorenim kamenitim siparima i visokoplaninskim pašnjacima u specifičnoj biljnoj zajednici. Predstavlja rubnu zajednicu šuma bora i obične bukve te u biljnoj zajednici planinskih pašnjaka,

forests and thickets, and coastal common beech forests [2]. Due to depopulation and the absence of human activity, open habitats heal, so the bushes disappear through the natural succession of vegetation. It is similar in Bosnia and Herzegovina, where its area is limited to the mountains Čvrsnica and Čabulja. It grows there on calcareous substrates, open rocky slopes and high mountain pastures in a specific plant community. It represents the marginal community of pine and common beech forests and in the plant community of mountain pastures, i.e. in specific ecological conditions.

Slika 1. **Hrvatska sibireja na planini Čabulji**
Figure 1. ***Croatian sibirea on Mount Čabulja***

odnosno u specifičnim ekološkim uvjetima.

Da bi više saznao o hrvatskoj sibireji, autor (D. Ballian, *op. ur.*) se obratio poznatom dendrologu i botaničaru dr. sc. Čedomilu Šiliću. Nažalost, od njega je malo saznao o nalazištima sibireje, osim da su teško dostupna, ali je doznao nešto vrlo zanimljivo. U Alpinetumu Šumarskog fakulteta u Sarajevu nalazili su se primjerci altajske sibireje (*Sibiraea altaiensis* (Laxm.) C. K. Schneid.), koje je Čedomil Šilić zasadio i morfološki uspoređivao, ali svoja opažanja nikada nije objavio. Došao je do zaključka da između hrvatske i altajske sibireje ne postoje morfološke razlike. Autora je ta Šilićeva konstatacija zaintrigirala, pa je o tome intenzivno razmišljao tijekom rada na doktorskoj disertaciji, za potrebe koje je molekularno-genetičkim metodama identificirao jedinke i populacije obične jele. Došao je na ideju da usporedi domaće sibireje s onima koje se nalaze na planini Altaj, smještenoj na granici Rusije, Kine i Kazahstana. U to vrijeme bilo je to jako smiono razmišljanje – prevladao je izazov da se usporede biljke koje su rasle na udaljenosti većoj od 5 000 km.

Vrsta *Sibiraea croatica* Degen, opisana je iz velebitskih populacija 1905., a godinu dana kasnije nađena je u Bosni i Hercegovini. Već 1927. dobiva novi status *Sibiraea altaiensis* (Laxm.) C. K. Schneider var. *croatica* (Degen) G. Beck [5], da bi se 1937. ponovno promijenio u *Sibiraea laevigata* (L.) Maxim., subsp. *croatica* (Degen) Degen. Inače, u Bosni i Hercegovini i Hrvatskoj hrvatska sibireja je zbog svoje rijetkosti u svojim prirodnim staništi-

In order to learn more about Croatian sibirea, the author (D. Ballian, *Ed. note)* turned to the famous dendrologist and botanist, Dr. Čedomil Šilić. Unfortunately, he learned little from him about the habitat of sibirea, except that they are difficult to access, but he learned something very interesting.

In the Alpinetum of the Faculty of Forestry in Sarajevo, there were specimens of Altaic siberia (*Sibiraea altaiensis* (Laxm.) C.K. Schneid.), which Čedomil Šilić planted and morphologically compared, but he never published his observations. He came to the conclusion that there are no morphological differences between Croatian and Altaic sibirea. The author was intrigued by Šilić's statement, so he thought about it intensively while working on his doctoral dissertation, for which he identified individuals and populations of common fir using molecular genetic methods. He came up with the idea to compare native sibireas with those found on the Altai Mountains, located on the border of Russia, China and Kazakhstan. At that time, it was a very courageous thought – he overcame the challenge of comparing plants that grew more than 5 000 km distant.

The species *Sibiraea croatica* Degen was described from Velebit populations in 1905, and a year later it was found in Bosnia and Herzegovina. As early as 1927, it received the new status of *Sibiraea altaiensis* (Laxm.) C. K. Schneider var. *croatica* (Degen) G. Beck [5], to be changed again in 1937 to *Sibiraea laevigata* (L.) Maxim., subsp. *croatica* (Degen) Degen. In Bosnia and Herzegovina and Croatia, due to its rarity in natural habitats, the Croatian sibirea has been protected by law since 1964, and since 1997 it has been on the red list of protected plant species (IUCN Red List of Threatened Plants) and

ma zakonom zaštićena od godine 1964., a od 1997. nalazi se na crvenom popisu zaštićenih biljnih vrsta (IUCN Red List of Threatened Plants) te dolazi u red zaštićenih endema. Primjerci hrvatske sibireje mogu se naći u nekim dendrološkim zbirkama i botaničkim vrtovima mnogih europskih zemalja.

Tijekom prikupljanja uzoraka za analizu autor saznaje da se altajska sibireja može pronaći na nekoliko lokaliteta: oko 1 500 km zapadno od Altaja, u južnoj Rusiji (Lipetska oblast) gdje je umjetno unesena, dok je u prirodnim staništima središnje Azije udaljena više od 5 000 km od dinarskih populacija. Na Altaju, u njegovom srednjem i zapadnom planinskom masivu raste na oko 1 300 m nadmorske visine.

Postoji još jedna zanimljiva vrsta u zapadnoj Kini u masivu Tianshan, tiansanska sibireja (*S. tianschanica* (Krassn.) A. Porjak), ali zbog nedostupnosti materijala nije bila uključena u istraživanje. Prema Kuminovoj [6] sibireja formira specifične biljne zajednice unutar visoke stepe i šumostepe na Altaju. Obično raste na otvorenim prostorima i traži povišene dijelove mikroreljefa visoke vlažnosti. Najveća preprjeka prirodnom održanju populacija sibireje su snažni antropogeni i zoogeni utjecaji, prije svega ekstenzivno stočarstvo.

Na temelju istraživanja koje su proveli Ballian i sur. [7-11], te Grebenc i sur. [12] staru taksonomiju, odnosno sistematiku sibireja trebalo je na odgovarajući način revidirati. Prema nekim literaturnim podatcima rod *Sibiraea* obuhvaća pet vrsta u europskom dijelu Južne Rusije, Sibira, is among the protected endemic. Specimens of Croatian sibirea can be found in some dendrological collections and botanical gardens of many European countries.

During the collection of samples for analysis, the author found out that the Altaic sibirea can be found in several localities: about 1 500 km west of Altai, in southern Russia (Lipetska region), where it was brought by plantation, while in the natural habitats of Central Asia, it is more than 5 000 km away from the Dinaric population. In Altai, in its central and western mountain massif, it grows at about 1 300 m above sea level.

There is another interesting species in western China in the Tianshan massif, the Tianshan sibirea (*S. tianschanica* (Krassn.) A. Porjak), but due to the unavailability of material, it was not included in the research. According to Kuminova [6], sibirea forms specific plant communities within the high steppe and forest-steppe in Altai. It usually grows in open areas and looks for elevated parts of microrelief with high humidity. The biggest obstacle to the natural maintenance of populations of sibirea is strong anthropogenic and zoogenic influences, primarily extensive animal husbandry.

Based on research completed by Ballian *et al.* [7-11], and Grebenc *et al.* [12] the old taxonomy, that is, the systematics of sibireas, had to be revised accordingly. According to some literature data, the genus *Sibiraea* includes five species in the European part of South Russia, Siberia, Western China and Southern Europe. However, Krüssmann [13] lists two species: with *S. laevigata* (L.) Maxim. (= *S. altainensis* Schneid.) and *S. tomentosa* Diels, with the distinction between two varieties within the first (*var. angusta* and *var. croatica*), which was confirmed by Ballian *et al.* [8]. They al-

Zapadne Kine i Južne Europe. Međutim, Krüssmann [13] navodi dvije vrste: sa *S. laevigata* (L.) Maxim. (= *S. altainensis* Schneid.) i *S. tomentosa* Diels, s time da unutar prve razlikuje dva varijeteta (*var. angusta* i *var. croatica*) što potvrđuju Ballian i sur. [8]. Također su istaknuli veliku taksonomsku bliskost koja ide čak do razine ekotipova, što nije pravi taksonomski status.

Rezultati istraživanja pokazali su da zbog velike genske sličnosti hrvatsku i altajsku sibireju ne treba shvaćati kao dvije odvojene vrste, već je riječ o podvrstama ili čak ekotipovima.

MATERIJALI I METODE

Osobnim kontaktom s dr. V. Melnikom pribavljeni su uzorci altajskih sibireja, dok su uzorci autohtonoga materijala sakupljeni na planini Čabulja u Bosni i Hercegovini i planini Velebit u Hrvatskoj (tablica 1). Osim uzoraka s Altaja, podrijetlom iz okolice Barnaula u Južnom Sibiru, uzorci su stigli i iz Lipetske oblasti u južnoj Rusiji. Za analizu su tijekom rujna 2003. prikupljene grančice s pupovima u početnoj fazi mirovanja, te na kraju vegetacijske dormancije u veljači 2004. Uzorci biljaka na svim lokalitetima sabirani su na međusobnoj udaljenosti najmanje 20 m.

DNA je ekstrahirana iz mirujućih pupova nakon uklanjanja suberiniziranih ovojnih listova pupova (tegmata) ili iz unutarnjeg dijela jezgre stabljike pomoću Plant DNeasy Mini Kit (Qiagen). Zatim je DNA resuspendirana u prethodno zagrijanoj, sterilnoj milli-Q vodi do približne konačne koncentracije od 100 ng/μL.

so pointed out a great taxonomic closeness that goes all the way to the level of ecotypes, which is not a true taxonomic status.

The results of the research showed that due to the great genetic similarity, Croatian and Altaic sibireas should not be understood as two separate species, but rather subspecies or even ecotypes.

MATERIALS AND METHODS

Through personal contact with Dr. V. Melnik, samples of Altaic sibirea were obtained, while samples of autochthonous material were collected on Mount Čabulja in Bosnia and Herzegovina and Mount Velebit in Croatia (*Table 1*). In addition to samples from Altai, originating from the Barnaul area in Southern Siberia, samples also arrived from the Lipetska region in southern Russia. During September 2003, twigs with buds were collected in the initial phase of rest, and at the end of vegetation dormancy in February 2004. Plant samples in all localities were collected at a distance of at least 20 m from each other.

DNA was extracted from dormant buds after removing the suberinized bud sheaths (tegmata) or from the inner part of the stem core using the Plant DNeasy Mini Kit (Qiagen). The DNA was then resuspended in pre-warmed, sterile milli-Q water to an approximate final concentration of 100 ng/μL. The quality and quantity of DNA were checked after separation in a 1% agarose gel stained with ethidium bromide.

Several different pairs of primers (*Table 2*) and programs were used on conducting DNA amplification, because previously there were no data on the use of polymerase chain reaction (PCR) with species from the genus *Sibiraea* [8]. Work was

Tablica 1. **Istraživane populacije i lokaliteti uzoraka sibireje** [8]
Table 1. ***Researched populations and localities of sibireas samples*** [8]

Populacija *Population*	Lokacija *Locality*	Longituda *Longitude*	Latituda *Latitude*	Altituda *Altitude*	Broj uzoraka *Number of samples*
Čabulja, Bosna i Hercegovina / *Bosnia and Herzegovina*	Rosne poljane	43°51'42" 43°54'85"	17°49'60"4 17°54'92"7	1 343 m 1 427 m	33
Velebit, Hrvatska / *Croatia*	Mali Brizovac (Velinac)	44°56'18" 44°56'24"	15°07'40"7 15°07'51"4	992 m 1 011 m	33
Lipetska oblast, Rusija / *Lipetska Region, Russia*	Stanovlianski raion	52°30'	39°32'	300 m	3
Altai, Rusija / *Russia*	Barnaul	85°37'	49°28'	do / *to* 1 300 m	5

Kakvoća i količina DNA provjerene su nakon odvajanja u 1 %-tnom agaroznom gelu obojenom etidijevim bromidom.

Pri provođenju amplifikacije DNA uporabljeno je nekoliko različitih parova početnica (tablica 2) i programa jer ranije nije bilo podataka o primjeni lančane reakcije polimerazom (PCR) s vrstama iz roda *Sibiraea* [8]. Radilo se na određenim specifičnim ITS regijama u genomskoj DNA i dijelovima kloroplastne DNA. Potonje početnice odabrane su s popisa početnica uporabljenih za amplifikaciju duhana (*Nicotiana tabacum*). Reakcije umnožavanja provedene su dvjema metodama: a) standardnim postupkom koji su opisali White i sur. [14] u ukupnom reakcijskom volumenu od 40 µL s AmplyTaq polimerazom (Perkin Elmer, Foster City, CA, SAD) za umnožavanje ITS regije u genomskoj DNA, i/ili b) modi-

done on certain specific ITS regions in genomic DNA and parts of chloroplast DNA. The latter primers were selected from the list of primers used for the amplification of tobacco (*Nicotiana tabacum*). Amplification reactions were carried out by two methods: a) with the standard procedure described by White *et al.* [14] in a total reaction volume of 40 µL with AmplyTaq polymerase (Perkin Elmer, Foster City, CA, USA) to amplify the ITS region in genomic DNA, and/or b) a modified general method for amplifying some primers (STS) in spruce [15] in a final volume of 25 µL using AmplyTaq Gold hot start polymerase (Applied Biosystems).

Ballian *et al.* [8] completed the polymerase chain reaction on a PE 9700 DNA thermocycler. Cycle parameters for ITS amplification with ITS primers were used according to the research conducted by Grebenc *et al.* [16]. Parameter cycles for cpDNA amplification included an initial denaturation at 95 °C

ficiranom općom metodom za umnažanje nekih početnica (STS) u smreke [15] u konačnom volumenu od 25 μL primjenom AmplyTaq Gold hot start polimeraze (Applied Biosystems).

Ballian i sur. [8] su lančanu reakciju polimerazom proveli na PE 9700 DNK termocikleru. Parametri ciklusa za ITS amplifikaciju s ITS početnicama uporabljeni su prema istraživanju koje su proveli Grebenc i sur. [16]. Parametri ciklusa za umnožavanje cpDNA uključivali su početnu denaturaciju pri 95 °C tijekom 5 minuta, nakon čega je slijedilo 40 ciklusa pri 94 °C tijekom jedne minute, zagrijavanja do 55 °C (53 °C – 63 °C kad je obavljeno optimiranje) tijekom dvije minute, i produljenje pri 72 °C tijekom tri minute, s konačnim produljenjem pri 72 °C tijekom 10 minuta i pohranjivanjem reakcije pri 4 °C. Kontrole bez DNA pokrenute su za svaki eksperiment kako bi se provjerila kontaminacija reagensa. Nisu izvedene pozitivne kontrole jer materijal za pozitivnu kontrolu nije bio dostupan, osim za par početnica ITS1 i ITS4 gdje je gljivična DNA služila kao pozitivna kontrola reakcije.

U provedenom istraživanju amplifikacijski produkti razdvojeni su elektroforezom na agaroznim gelovima koji sadrže 2 % LE agaroze (SeaKem, Rockland, MA, SAD) u 1 x TBE puferu jedan sat pri naponu 5 Vcm^{-1} (Ballian i sur., [8]). DNA je obojena etidijevim bromidom, vizualizirana pod UV-svjetlom i snimljena na Polaroid 667 BW film. Duljine amplifikacijskih produkata procijenjene su usporedbom s GeneRuler™ 100 bp DNA Ladder (Fermentas).

for 5 min, followed by 40 cycles at 94 °C for 1 min, heating to 55 °C (53 °C – 63 °C when optimized) for two minutes, and extension at 72 °C for three minutes, with a final extension at 72 °C for 10 minutes and storage of the reaction at 4 °C. DNA-free controls were made for each experiment to check for reagent contamination. No positive controls were performed because positive control material was not available, except for the primer pair ITS1 and ITS4 where fungal DNA served as a positive control for the reaction.

In the executed research, the amplification products were separated by electrophoresis on agarose gels containing 2 % LE agarose (SeaKem, Rockland, MA, USA) in 1 x TBE buffer for one hour at a voltage of 5 Vcm^{-1} (Ballian *et al.*, [8]). DNA was stained with ethidium bromide, visualized under UV light and recorded on Polaroid 667 BW film. The lengths of the amplification products were estimated by comparison with the GeneRuler™ 100 bp DNA Ladder (Fermentas).

Amplification products that yielded only one pure fragment of the expected length (as estimated from the length of the products from *Nicotiana tabacum* and ectomycorrhizal fungi) were digested separately with one unit of each restriction enzyme according to the manufacturer's recommendations in a total volume of 10 μL. Amplified ITS regions in genomic DNA were excised using Hinf I (Promega, Madison, WI, USA), Mbo I (Promega, Madison, WI, USA), and Taq I (TaKaRa, Shiga, Japan). The cpDNA amplification products were cut with Hinf I (Promega, Madison, WI, USA), Hae III (Fermentas), and Alu I (Fermentas; TaKaRa) enzymes. Enzymes were selected based on economic criteria (for cpDNA) and follow-

Amplifikacijski produkti koji su dali samo jedan čisti fragment očekivane duljine (kako je procijenjeno na temelju duljine produkata iz vrste *Nicotiana tabacum* i ektomikoriznih gljiva) digestirani su odvojeno s jednom jedinicom svakoga restrikcijskog enzima prema preporukama proizvođača u ukupnom volumenu od 10 μL. Umnožene ITS regije u genomskoj DNA izrezane su pomoću Hinf I (Promega, Madison, Wi, SAD), Mbo I (Promega, Madison, Wi, SAD) i Taq I (TaKaRa, Shiga, Japan). Amplifikacijski produkti cpDNA izrezani su Hinf I (Promega, Madison, Wi, SAD), Hae III (Fermentas) i Alu I (Fermentas; TaKaRa) enzimima. Enzimi su odabrani na temelju ekonomskih kriterija (za cpDNA) i nakon prethodno objavljenih sekvenci ITS regije [17]. Restrikcijski fragmenti su odvojeni na 2 %-tnom agaroznom gelu u TBE puferu tijekom tri sata pri 10 Vcm^{-1}. Duljina restrikcijskih fragmenata procijenjena je usporedbom s GeneRuler™ 100 bp DNK Ladder (Fermentas).

Analizirani gelovi su obojeni u etidijevu bromidu, dobiveni sustavom GelDoc (Biorad) i analizirani softverom Taxotron® (Institut Pasteur 1998., Pariz, Francuska) [18]. Postupak su detaljno opisali Grebenc i sur. [16] i Martín i Kårén [19].

Prije slanja na sekvenciranje, amplifikacijski produkti su očišćeni uporabom Wizard SV Gel i PCR Clean-Up System (Promega). Oba lanca amplificirane DNA sekvencirana su zasebno uporabom već spomenutih početnica. Ukoliko su produkti amplifikacije bili nedovoljni

ing previously published sequences of the ITS region [17]. Restriction fragments were separated on a 2 % agarose gel in TBE buffer for three hours at 10 Vcm^{-1}. The length of the restriction fragments was estimated by comparison with the GeneRuler™ 100 bp DNA Ladder (Fermentas). The analyzed gels were stained with ethidium bromide, obtained with the Gel-Doc system (Biorad) and analyzed with the Taxotron® software (Pasteur Institute 1998, Paris, France) [18]. The procedure was described in detail by Grebenc *et al.* [16] and Martín and Kårén [19].

Before sending for sequencing, amplification products were cleaned using Wizard SV Gel and PCR Clean-Up System (Promega). Both strands of the amplified DNA were sequenced separately using the previously mentioned primers. If the amplification products were insufficient or more than one was produced, before sequencing, products of the desired length were purified from the gel using the same purification kit. DNA was sequenced using a commercially available method (Sequiserve, Germany). A total of eight samples was sequenced, three from each population in Croatia and Bosnia and Herzegovina, two from the Altai population and one from the Lipetska region. A part of genomic DNA was sequenced using ITS5-p and ITS4 primers and parts of cpDNA using ccmp 10-R, trn HM, trn G-P and trn MM primers.

Sequence Navigator software (Applied Biosystems) was used to identify the specific sequences from the two strands of each isolate. Dialign 2 software was used for multiple alignment of the obtained sequences [20] with some minor manual corrections. The new sequences are deposited in the EMBL database with accession

ili je nastalo više od jednog, prije sekvenciranja su produkti željene duljine očišćeni od gela istim priborom za pročišćavanje. DNA je sekvencirana komercijalno dostupnom metodom (Sequiserve, Njemačka). Sekvencirano je ukupno osam uzoraka, po tri iz svake populacije u Hrvatskoj i Bosni i Hercegovini, dva iz populacije Altaja i Lipetske oblasti (tablica 1). Sekvencirani su dio genomske DNA s pomoću ITS5-p i ITS4 početnica i dijelovi cpDNA s pomoću ccmp 10-R, trn HM, trn G-P i trn MM početnica.

Softver Sequence Navigator (Applied Biosystems) služio je za identifikaciju određene sekvencije iz dva lanca svakog izolata. Softver Dialign 2 korišten je za višestruko poravnavanje dobivenih sekvencija [20] uz neke manje ručne korekcije. Nove sekvencije pohranjene su u bazi podataka EMBL s pristupnim brojevima, kao i pristupnim brojevima sekvencija objavljenih u bazi podataka GenBank za odabrane predstavnike obitelji *Rosaceae*.

REZULTATI

Za analizu i potvrdu taksonomskog statusa hrvatske sibireje (*Sibiraea croatica* Degen), uporabljeno je ukupno 16 početnica, koje su kombinirane u devet mogućih parova početnica (tablica 2). Nakon što su provedene sve analize dobiveni rezultati poslije amplifikacije pokazali su da postoji samo jako mala ili nikakva varijabilnost u duljini fragmenata amplificirane DNA.

Nakon što su uspješno završene lančane reakcije polimerazom dobiveni amplinumbers as well as the accession numbers of the sequences published in the GenBank database for selected representatives of the *Rosaceae* family.

RESULTS

For the analysis and confirmation of the taxonomic status of the Croatian sybirea (*Sibiraea croatica* Degen), a total of 16 primers were used, which were combined into nine possible pairs of primers (*Table 2*). The analyses were performed and the post-amplification results showed that there was very little or no variability in the length of the amplified DNA fragments.

After the polymerase chain reactions were successfully completed, the obtained amplificates were cut with restriction enzymes (*Table 3*), and confirmed that there are no differences between and within the studied sibireas population that could be detected on the agarose gel. Because of this, sequencing was undertaken, but it also confirmed only a very small variability between populations. The amplified product using the trn GP and trn MM primer pairs showed no differences within the 247 bp long sequence. By applying BLAST [21], i.e. an algorithm for comparing information on primary biological sequences, the results of chloroplast DNA sequencing showed a significant similarity of the trnG-trnM region for intergenic distances as in *Prunus zippeliana* species (E = 127, bit value = 1e-26, GenBank acc. no. = AB111589). The sequences obtained with ccmp 10-R and reverse primer trn HM were 274 to 280 bp long with a 6 bp duplication in the form of GTATAT of 215 bp towards the 5' end. Duplication exists in all samples from Vel-

fikati su izrezani restrikcijskim enzimima (tablica 3), te su potvrdili da između i unutar proučavanih populacija sibireja nema razlika koje bi se mogle detektirati na agaroznom gelu. Zbog toga se pristupilo sekvencioniranju, ali je i ono potvrdilo samo jako malu varijabilnost između populacija. Umnoženi proizvod uporabom trn GP i trn MM para početnica nije pokazao razlike unutar 247 bp duge sekvencije. Primjenom BLAST-a [21], ebit and one sample from Altai. Both sequences gave an identical result after BLAST [21] and were similar to *Vigna angularis* and its chloroplast S10B operon (E = 92, bit value = 7e-16, GenBank acc. no. = AF536225). The insertion that appeared there was 6 bp long, and was not detected earlier on agarose gels due to the inaccurate method.

The ITS region in genomic rDNA was sequenced for the same samples. All nine sequences were 762 bp long for ITS5-p and ITS4 prim-

Tablica 2. **Pregled početnica korištenih u istraživanju; T_m – temperatura žarenja** [8]
Table 2. ***Overview of primers used in the research; T_m – annealing temperature*** [8]

Ime početnice *Primer name*	Ime sekvencije *Sequence name*	T_m / °C	Literatura *References*
ITS1 (nuclear)	5'- TCC GTA GGT GAA CCT GCG G -3'	64	14
ITS4 (nuclear)	5'- TCC TCC GCT TAT TGA TAT GC -3'	58	14
ITS5 (nuclear)	5'- GGA AGT AAA AGT CGT AAC AAG G -3'	59	16
ITS5-p (nuclear)	5'- GGA AGG AGA AGT CGT AAC AAG -3'	61	17
atpB-1 (cpDNA)	5'- ACA TCK ART ACK GGA CCA ATA A -3'	58	18
rbcL-1 (cpDNA)	5'- AAC ACC AGC TTT RAA TCC AA -3'	55	18
ccmp10-R (cpDNA)	5'- TTC GTC GDC GTA GTA AAT AG -3'	57	18
trnHM (cpDNA)	5'- GTG AAT CCA CCA YGC GCG GG -3'	68	http://fbva.forvie.ac.at/200/ 2043.html
trnG-P (cpDNA)	5'- GCC AAG GAG AAG ATG CGG G -3'	65	20
trnMM (cpDNA)	5'- CAT AAC CTT GAG GTC ACG GG -3'	62	21
trnC-f (cpDNA)	5'- CCA GTT CAA ATC TGG GTG TC -3'	60	21
trnD-m (cpDNA)	5'- GGG ATT GTA GTT CAA TTG GT -3'	56	21
ycf9-P (cpDNA)	5'- TTG CYT CTC CYG ATG GYT GG -3'	63	http://fbva.forvie.ac.at/200/ 2043.html
rps 14 (cpDNA)	5'- CTA TCC GGA CAC ATA CTT CG -3'	60	22
trnT (cpDNA)	5'- CAT TAC AAA TGC GAT GCT CT -3'	56	23
trnF (cpDNA)	5'- ATT TGA ACT GGT GAC ACG AG -3'	58	23

odnosno algoritma za usporedbu informacija o primarnim biološkim sekvencijama, rezultati sekvencioniranja kloroplastne DNA pokazali su značajnu sličnost regije trnG-trnM za intergenske razmake kao kod vrste *Prunus zippeliana* (E = 127, vrijednost bita = 1e-26, GenBank acc. no. = AB111589). Sekvencije dobivene sa ccmp 10-R i reverznom početnicom trn HM bile su duge 274 do 280 bp sa 6 bp podvostručenja u obliku GTATAT od 215 bp prema 5' kraju. Duplikacija postoji u svim uzorcima s Velebita i jednom uzorku s Altaja. Obje sekvencije dale su identičan rezultat nakon BLAST-a [21] i bile su slične kao kod vrste *Vigna angularis* odnosno njenom kloroplastnom S10B operonu (E = 92, vrijednost bita = 7e-16, GenBank acc. no. = AF536225). Insercija koja se tu pojavila bila je duga 6 bp, te nije otkrivena ranije na agaroznim gelovima zbog nepreciznosti metode.

ITS regija u genomskoj rDNA sekvencirana je za iste uzorke. Svih devet sekvencija bile su duge 762 bp za početnice ITS5-p i ITS4. Nizovi su pokazali netočnost kod nekih baza blizu 5' kraja neovisno o podrijetlu (populaciji) uzorka. Na tim mjestima u ITS regija u genomskoj rDNA sekvencirana je za iste DNA nizu određene se baze nisu mogle pravilno očitati jer su dvije baze davale približno jednaki intenzitet signala. Sve sekvencirane ITS regije sibireja uključene su u filogenetsku analizu s odabranim vrstama iz porodice ružovki (*Rosaceae*) koje su dostupne u bazi podataka GenBank. Tako je za sve rodove korištene u filogenetskoj usporedbi s novo dobivenim sekvencijama, ers. The arrays showed imprecision at some bases near the 5' end regardless of the origin (population) of the sample. At those places in the DNA sequence, certain bases could not be read properly because the two bases gave approximately equal signal intensity. All sequenced ITS regions of sibireas were included in the phylogenetic analysis with selected species from the *Rosaceae* family available in the GenBank database. This is the case for all genera used in the phylogenetic comparison with newly obtained sequences, and for sibirea sequences were taken from available databases with their references from 2005 (*Table 4*).

DISCUSSION

The origin of analyzed disjuncted populations of sibireas

Earth's evolution is in constant motion, and thus the species as well as the ecosystems in which they occur. Thus, throughout its history, sibirea was constantly exposed to changes. During the Tertiary period, larger areas of Southeastern Europe, Southern Siberia and Western China had different ecological characteristics and vegetation cover. The southern part of Europe and Siberia were covered with steppes and forest-steppes [22,23], and they reached their maximum distribution during the Quaternary. They then spread from the Adriatic Sea to Eastern Siberia with their characteristic flora elements, which can still be found today in isolated areas of Southern Siberia, where sibirea also grew. Kuminova [6], in her research in the East of Russia, in the Southwest of Siberia, described specific plant communities of the high steppe and steppe forests. In these plant communities, she cites sibirea from the Altai Mountains as one of the most important species, so it can be said that during the

Tablica 3. **PCR amplifikacija i duljina restrikcijskih fragmenata [bp] za sve pozitivne reakcije iz različitih populacija [8]; početnice su označene uobičajenim nazivima**

Table 3. ***PCR amplification and length of restriction fragments [bp] for all positive reactions from different populations [8]; common names for the primers were used***

Parovi početnica / *Primer pairs*	Kakvoća i duljina PCR proizvoda / *PCR quality and length of product [bp]*	Restrikcijski fragmenti PCR proizvoda [bp] *Restriction fragments of the PCR products [bp]*				
		Hinf I	Mbo I	Taq I	Alu I	Hae III
ITS1 ITS4	slaba amplifikacija / *weak amplification*	_*	_	_	_	_
ITS5 ITS4	višestruke vrpce / *multiple bands*	_	_	_	_	_
ITS5-p ITS4	773	173, 157, 151, 128, 83, 67	582, 160	347, 260, 75, 60	_	_
ccmp10-RtrnHM	274 (280)**	140, 127, 7 (146, 127, 7)**	274(280)** (bez restrikcije / *no restriction*)	_	274 (280)** (bez restrikcije / *no estriction*)	274(280)** (bez restrikcije / *no restriction*)
trnG-P trnMM	240	240 (bez restrikcije / *no restriction*)	240 (bez restrikcije / *no restriction*)	_	240 (bez restrikcije / *no restriction*)	240 (bez restrikcije / *no restriction*)
trnC- ftrn	višestruke vrpce / *multiple bands*	_	_	_	_	_
atpB-1 rbcL-1	bez amplifikacije / *no amplification*	_	_	_	_	_
trnT-trnF	slaba amplifikacija / *weak amplification*	_	_	_	_	_
trnG-P trnMM	240	240 (bez restrikcije / *no restriction*)	240 (bez restrikcije / *no restriction*)	_	240 (bez restrikcije / *no restriction*)	240 (bez restrikcije / *no restriction*)
trnC-f trnD-m	višestruke vrpce / *multiple bands*	_	_	_	_	_

(–)* Reakcija nije provedena / *Reaction not performed.*

** Za uzorke iz populacije Velebit i nekoliko uzoraka iz populacije Altai. / *For samples from population Velebit and a few samples from population Altai.*

a za sibireje su preuzete sekvencije iz dostupnih baza podataka s njihovim referencijama iz 2005. godine (tablica 4).

RASPRAVA

Podrijetlo analiziranih disjunktnih populacija sibireje

Zemljina je evolucija u stalnom kretanju, a samim time i vrste kao i ekosustavi u kojima se javljaju. Tako je i sibireja bila kroz svoju povijest stalno izložena promjenama. Tijekom tercijara veća područja jugoistočne Europe, južnog Sibira i zapadne Kine imala su drugačija ekološka obilježja i vegetacijski pokrov. Južni dio Europe i Sibira bili su prekriveni stepama i šumostepama [22,23], da bi one tijekom kvartara dosegle svoju maksimalnu rasprostranjenost. One se tada prostiru od Jadranskog mora do istočnog Sibira s njihovim karakterističnim flornim elementima, koji se i danas mogu naći u izoliranim područjima južnog Sibira, gdje je rasla i sibireja. Kuminova [6] je u svojim istraživanjima na istoku Rusije, na jugozapadu Sibira, opisala specifične biljne zajednice visoke stepe i stepskih šuma. U tim biljnim zajednicama navodi sibireju s planine Altaj kao jednu je od najznačajnijih vrsta, pa se može reći da je tijekom kvartara i tercijara bila široko rasprostranjena na čitavom tom prostoru.

Krajem tercijara klima se mijenja, a nakon velikih klimatskih promjena u glacijalnom razdoblju, populacije sibireje su razdvojene u male i udaljene izolirane otoke, tj. u disjunktna područja. Jugoistočnoeuropske populacije sibireje ostale su izolirane na planinama Velebit, Čabulja i Čvrsnica, obuhvaćajući male subpopulaci-

Tablica 4. **Dostupne sekvencije** [8]
Table 4. ***Available sequences*** [8]

Agrimonia eupatoria L., U90798
Aremonia agrimonoides (L.) D. C., U90799
Cotoneaster lacteus W. W. Smith., U16188
Crataegus mollis (Torr. & A. Gray) Scheele, U16190
Cydonia oblonga Mill., AF186531
Dryas octopetala L., U90804
Fragaria moschata Duchesne, AF163505
Fragaria vesca L., U90793
Fragaria vesca subsp. *vesca* forma *alba* (Ehrh.) Staudt., AF163516
Fragaria vesca L. subp. *vesca*, AF163515
Fragaria x ananassa Duchesne, AF164494
Filipendula ulmaria (L.) Maxim., U90783
Filipendula vulgaris Moench, AJ416467
Geum montanum L., AJ302350
Geum rivale L., AJ302352
Malus sieversii (Ledeb.) M. Roem., AF186491
Malus x *domestica* Borkh., U16195
Mespilus germanica L., U16196
Potentilla dickinsii Back., U90785
Potentilla fruticosa L., AF163478
Prunus armeniaca L., AF318756
Prunus avium L., AF318737
Prunus cerasus L., AF318729
Prunus domestica L., AF318713
Prunus persica (L.) Batsch, AF318741
Pyrus pyrifolia (Burm. f.) Nakai., AF287240
Rosa canina L., AB019495
Rosa persica Michx, AJ416468
Rubus caucasicus Focke, AY083371
Rubus idaeus L., AF055756
Rubus neomexicanus Gray, AY083358
Sanguisorba officinalis L., AY635040
Sorbus aucuparia L., U16204
Sorbus torminalis (L.) Crantz., AF086533
Spiraea cantoniense Lour, AF318722
Spiraea x *vanhouttei* (Briot) Zabel, U16205
Waldsteinia geoides Willd., AJ302362

je s relativno malim brojem jedinki. U Hrvatskoj se grmovi sibireje javljaju u biljnoj zajednici *Seslerio-Ostryetum* Horvat [2], a u Bosni i Hercegovini u zasebnim biljnim zajednicama u visokim planinskim predjelima na krševitim vapnenačkim terenima. Iako još nisu potpuno istražene, svojom morfologijom i otvorenošću staništa djelomično podsjećaju na šumostepe. Analizirani su i uzorci iz južne Rusije, s lokaliteta na kojima je sibireja umjetno unesena u svrhu izrade rukotvorina ili zaštite tla od erozije. Stoga sadašnja rasprostranjenost sibireje u jugoistočnoj Europi i južnom Sibiru najvjerojatnije predstavlja samo ostatke početnoga velikoga područja rasprostranjenosti iz razdoblja tercijara i kvartara. Sadašnje područje rasprostranjenosti predstavlja glacijalni refugij nastao tijekom posljednje glacijacije. Međutim, paleontološke analize fosilnog polena nisu dostupne da bi dokazale tu pretpostavku, ali postoje analize peludi sa živog materijala s područja Velebita [24].

Polimorfizam cpDNA i ITS bio je minimalan ili nije otkriven

Analizirana nekodirajuća područja u cpDNA pokazuju sporu stopu evolucije i mogu se koristiti za diferencijaciju između vrsta i populacija [25]. U ovom istraživanju korišteno je šest pari početnica za amplifikaciju dijelova nekodirajućih regija u cpDNA; opće početnice napravljene za amplifikaciju atpB-rbcL gena kod mahunarki [26] i početnice napravljene za duhan (tablica 2). Odabrane početnice nisu dizajnirane posebno za lančanu reakciju polimerazom kod sibireja niti su modifi-

Quaternary and Tertiary it was widely distributed throughout that area.

At the end of the Tertiary and after major climatic changes in the glacial period, the populations of sibireas were separated into small and distant isolated islands, i.e. into disjuncted areas. The Southeast European populations of sibirea remained isolated in the Velebit, Čabulja and Čvrsnica mountains, comprising small subpopulations with a relatively small number of individuals. In Croatia, sibirea shrubs occur in the plant community *Seslerio-Ostryetum* Horvat [2], and in Bosnia and Herzegovina in separate plant communities in high mountain areas on rugged limestone terrain. Although they have not yet been fully explored, their morphology and openness of habitats partially resemble forest-steppes. Samples from southern Russia, from localities where sibirea was artificially introduced for the purpose of making handicrafts or protecting the soil from erosion, were also analyzed. Therefore, the current distribution of sibirea in Southeastern Europe and Southern Siberia most likely represents only the remains of the initial large area of distribution from the Tertiary and Quaternary periods. The current distribution area represents a glacial refugium created during the last glaciation. However, paleontological analyses of fossil pollen are not available to prove this assumption, but there are analyses of pollen of living material from the Velebit area [24].

cpDNA and ITS polymorphism was minimal or not detected

Analyzed non-coding regions in cpDNA show a slow rate of evolution and can be used to differentiate between species and populations [25]. In this study, six primer pairs were

cirane za tu priliku. Ipak, za analizu su optimirane PCR reakcije za određene parove početnica bez ili sa slabim pojačanjem. Umnožene DNA sekvencije nisu pokazale razlike između populacije u restrikcijskim obrascima, iako je dobro poznato da lančana reakcija polimeraze u kombinaciji s polimorfizmom duljine restrikcijskih fragmenata (PCR-RFLP) razdvaja većinu vrsta, ali i neke populacije [17,26,14]. U istraživanom slučaju je registrirana samo manja razlika u duplikaciji od 6 bp na umnoženoj cpDNA uz pomoć početnice ccmp 10R i njenim parom početnicom trnM. Udvostručivanje je vjerojatno rezultat jednostavnog umetanja u nekodirajuću regiju, a registrirano je u dvije zemljopisno različite populacije.

Kod restrikcijskog obrasca za ITS regiju u genomskoj rDNA nisu registrirane razlike, što ukazuje na to da svi uzorci sibireje pripadaju istoj vrsti jer je dokazano da je ITS regija očuvana unutar jedne taksonomske vrste. Uočene supstitucije ili netočnosti u sekvenciji iste regije dale su razliku 0-2 para baza između različitih sekvencija ITS-a, što je usporedivo s razlikama uočenim unutar kompleksa vrste *Fragaria vesca* gdje su registrirane razlike od 0-3 para baza. Dobivene gotovo identične sekvencije mogu ukazivati na nedavno razdvajanje i moguću specijaciju istraživanih populacija zbog njihove nedavne kolonizacije kao što je uočeno kod vrsta roda *Adenocarpus* [27]. Vogler i DeSalle [28] raspravljali su o tome da je glavna evolucijska sila kod ribosomske regije bila usklađena s tokom evolucije, koja bi trebala dovesti do homogenizacije

used to amplify parts of non-coding regions in cpDNA; general primers designed for amplification of the atpB-rbcL gene in legumes [26] and primers designed for tobacco (*Table 2*). The selected primers were not designed specifically for polymerase chain reaction in sibireas nor were modified for this purpose. However, PCR reactions for certain pairs of primers without or with weak amplification were optimized for analysis. Amplified DNA sequences did not show differences between populations in restriction patterns, although it is well known that polymerase chain reaction combined with restriction fragment length polymorphism (PCR-RFLP) separates most species, but also some populations [17,26,14]. In the investigated case, only a minor difference in duplication of 6 bp was registered on the amplified cpDNA with the help of primer ccmp 10R and its pair primer trnM. The duplication is probably the result of a simple insertion in a non-coding region, and has been registered in two geographically distinct populations.

No differences were registered in the restriction pattern for the ITS region in the genomic rDNA, which indicates that all samples of sibirea belong to the same species, as it has been proven that the ITS region is conserved within one taxonomic species. The observed substitutions or inaccuracies in the sequence of the same region gave a difference of 0-2 base pairs between different ITS sequences, which is comparable to the differences observed within the complex species *Fragaria vesca* where differences of 0-3 base pairs were registered. The almost identical sequences obtained may indicate a recent separation and possible speciation of the investigated popu-

pojedinačnih ponavljanja i proizvoditi uglavnom jednostruke sekvencije u svim ponavljanjima određene vrste. Usklađena evolucija mogla bi biti problem ako postoje slučajevi alopoliploidije, ali je predviđeno da su *Sibiraea* spp. diploidne. Osim toga, očuvanje ribosomskih gena (uključujući ITS regije) prikazano je i korišteno zajedno s maternalnim naslijeđenim u regijama cpDNA za filogenetsku analizu i definiciju vrsta kod brojnih biljnih skupina [29,30] kao i u drugim organizmima kao što su više gljive, gdje se mogu uočiti neke varijacije na razini morfoloških svojstava [17,26,31]. Varijabilnost unutar vrste mogla bi se objasniti netočnošću koja je uočena kod nekih dijelova ITS sekvencija. Točkaste mutacije također se mogu pojaviti i ostati fiksne unutar jedne vrste/uzorka što rezultira heterozigotnošću ITS regije (ili barem u nekim kopijama rDNA, a što u konačnici rezultira heterodupleksima). Postojanost takvih mutacija u populacijama može se objasniti malim, disjunktnim populacijama s velikom vjerojatnošću samooplodnje ili križanja u srodstvu, kao i vegetativnog razmnožavanja. Slična polimorfna nukleotidna mjesta objašnjena kao superpozicija dvaju ili više različitih ITS ponavljanja unutar jednog ili više ribosomskih genskih klastera također su primijećena i dokazano postoje u nekoliko taksona roda *Amelanchier*, a koji pokazuju uobičajenu aseksualnu proizvodnju sjemena. Ballian i sur. [8] su stoga predložili za sibireju četiri moguća razloga za postojanost polimorfizma: polimorfizam unutar jedinke kao posljedica

lations due to their recent colonization as observed in *Adenocarpus* species [27]. Vogler and DeSalle [28] argued that the main evolutionary force at the ribosomal region was aligned with the course of evolution, which should lead to the homogenization of individual repeats and produce mostly single sequences in all repeats of a given species. Coordinated evolution could be a problem if there are cases of allopolyploidy, but *Sibiraea* spp are predicted to be deployed. In addition, conservation of ribosomal genes (including ITS regions) has been demonstrated and used together with maternally inherited cpDNA regions for phylogenetic analysis and species definition in numerous plant groups [29,30] as well as in other organisms such as higher fungi, where they can observe some variations at the level of morphological properties [17,26,31]. The variability within the species could be explained by the inaccuracy observed in some parts of the ITS sequences. Point mutations can also occur and remain fixed within a species/specimen resulting in heterozygosity of the ITS region (or at least in some rDNA copies, ultimately resulting in heteroduplexes). The persistence of such mutations in populations can be explained by small, disjuncted populations with a high probability of self-fertilization or inbreeding, as well as vegetative propagation. Similar polymorphic nucleotide sites explained as the superposition of two or more different ITS repeats within one or more ribosomal gene clusters have also been observed and proven to exist in several taxa of the genus *Amelanchier*, which show normal asexual seed production. Ballian *et al.* [8] therefore proposed for sibirea four possible reasons for the persistence of polymor-

prijelaznog stadija u usklađenoj evoluciji, interspecifična hibridizacija, evolucija pseudogena i poremećaj usklađene evolucije zbog nehomolognog položaja lokusa rDNA [32].

Tu je zanimljivo napomenuti da je među nekoliko bližih srodnika sibireja bilo i nekoliko sekvenciranih vrsta iz roda *Spiraea*, te su potvrdili položaj roda *Sibiraea*, koji je utvrđen prema morfološkim karakteristikama unutar sekcije *Spireae* i podporodice *Malonideae* [33] i molekularno kroz istraživanje koje su proveli Potter i sur. [34].

U istraživanjima koja su provedena za vrste iz roda *Zelkova*, odnosno dvije endemične vrste, *Zelkova abelicea* (Lam.) Boisser i *Zelkova sucula* Di Pasquale, rezultati pokazuju da se razlikuju samo u jednom paru baza i to u trnL intronu i nije otkrivena nikakva raznolikost unutar populacije pomoću različitih kloroplastnih biljega [35]. Ti autori sugeriraju da je nedavno došlo do odvajanja vrsta njihova jasnijeg razgraničenja. Prema uočenoj sličnosti ITS1, 5.8S i ITS2 regiji u genomskim rDNA klasterima umnoženim iz populacija hrvatske sibireje prikupljenih na Čabulji (Bosna i Hercegovina) i Velebitu (Hrvatska) te altajske sibireje s oba lokaliteta, na Altaju i južnoj Rusiji, nije potvrđeno postojanje hrvatske sibireje (*S. croatica*) kao zasebne vrste. Međutim, nedostatak molekularnih dokaza za zadržavanje hrvatske sibireje kao zasebne vrste ne bi trebao umanjiti važnost prisutnosti sibireje za regionalnu floru u jugoistočnoj Europi (planine zapadnog Balkana) gdje je veoma rijetka vrsta, a na nekim lokalitetima joj prijeti potpuni nestanak.

phism: intra-individual polymorphism as a consequence of a transitional stage in concerted evolution, interspecific hybridization, evolution of pseudogenes and disruption of concerted evolution due to the non-homologous position of the rDNA locus [32].

It is interesting to note here that among several close relatives of sibirea there were also several sequenced species from the genus *Spiraea*, and they confirmed the position of the genus *Sibiraea*, which was established according to morphological characteristics within the section *Spiraea* and the subfamily *Malonideae* [33] and molecularly through research conducted by Potter *et al.* [34].

In research conducted for species from the Zelkova genus, i.e. two endemic species, *Zelkova abelicea* (Lam.) Boisser and *Zelkova sucula* Di Pasquale, the results show that they differ only in one pair of bases, namely in the trnL intron, and no diversity within populations using different chloroplast markers [35]. These authors suggest that recently there has been a separation of species with a clearer demarcation. According to the observed similarity of the ITS1, 5,8S and ITS2 region in the genomic rDNA clusters multiplied from populations of Croatian sibirea collected on Čabulja (Bosnia and Herzegovina) and Velebit (Croatia) and Altaic sibireas from both localities, in Altai and southern Russia, the existence of Croatian sibireas (*S. croatica*) is not confirmed as separate species. However, the lack of molecular evidence for the retention of Croatian sibirea as a separate species should not diminish the importance of the presence of sibirea for the regional flora in southeastern Europe (mountains of the Western Balkans), where it is a very rare spe-

Brojni evolutivni procesi koji upravljaju prirodnim selekcijama u populacijama i zbog mutacijskih promjena u malim disjunktnim populacijama koje bi dovele do diferencijacije populacija, odnosno vrsta, još nisu dobro poznati [36]. Kako fenomen prirodne selekcije djeluje slično u malim izoliranim populacijama može se ilustrirati i na primjeru vrste *Abies nebrodensis* (Lojac.) Mattei, gdje selekcija daje prednost samo heterozigotima [37]. Sličan je fenomen zabilježen i za populacije vrste *Pinus leucodermis* Ant. kako izvještavaju Boscherini i sur. [38], a i u podpopulacijama *Picea abies* (L.) Karst. u ekstremnim uvjetima rasta [39,40].

ZAKLJUČAK

Genska struktura hrvatske sibireje u području zapadnog Balkana i njen odnos sa sibirejom iz južne Rusije i južnog Sibira analizirani su s pomoću amplifikacije, restrikcije i sekvencioniranja ITS regije u genomskoj i kloroplastnoj DNA te su uspoređene sa sekvencijama drugih vrsta iz porodice *Rosaceae*, a koje su preuzete iz baze podataka GenBank.

Restrikcijska analiza i odvajanje u agaroznom gelu nisu pokazali razlike u duljini digestirane cpDNA između ili unutar populacija. Sekvenciranje je pokazalo samo manju varijabilnost kod ispitanih populacija. Primijećena je samo manja razlika udvostručenja od 6 bp u DNA umnoženoj s ccmp 10 R i parom početnica trnM. Udvostručivanje bi moglo biti rezultat jednostavnog umetanja u nekodirajuću regiju i primijećeno je u dvije geografski različite populacije.

cies, and in some localities it is threatened with complete disappearance.

Many evolutionary processes that govern natural selection in populations and mutational changes in small disjunct populations that would lead to the differentiation of populations, relating to species, are not yet well known [36]. How the phenomenon of natural selection acts similarly in small isolated populations can be illustrated by the example of the species *Abies nebrodensis* (Lojac.) Mattei, where selection favors only heterozygotes [37]. A similar phenomenon was recorded in populations of *Pinus leucodermis* Ant. as reported by Boscherini *et al.* [38], and in subpopulations of *Picea abies* (L.) Karst, in extreme growth conditions [39,40].

CONCLUSIONS

The genetic structure of the Croatian sibirea in the area of the Western Balkans and its relationship with the sibirea from Southern Russia and Southern Siberia were analyzed using amplification, restriction and sequencing of the ITS region in genomic and chloroplast DNA, and was compared with the sequences of other species from the *Rosaceae* family, which are taken from the GenBank database. Restriction analysis and agarose gel separation showed no differences in the length of digested cpDNA between or within populations. Sequencing showed only minor variability between investigated populations. Only a minor fold difference of 6 bp was observed in DNA amplified with comp 10 R and the trn/*M primer pair. The duplication could result from a simple insertion in a non-coding region and was observed in two geographically distinct populations.

Nema razlika u restrikcijskom obrascu za ITS regiju u genomskoj rDNA što ukazuje na to da svi uzorci sibireje pripadaju istoj vrsti jer je dokazano da je ITS regija očuvana unutar jedne taksonomske vrste. Uočene supstitucije ili netočnosti u sekvenciji iste regije dale su razliku 0-2 para baza između različitih sekvencija ITS-a.

Manje razlike dobivene na molekularnoj razini podupiru hipotezu da je sibireja stari tercijarni relikt koji pokazuje manju varijabilnost. To istraživanje potvrdilo je Šilićeve prethodne preliminarne rezultate koje je osobno prenio autoru (D. Ballian, *op. ur.*), gdje su rađene usporedbe hrvatskih i altajskih sibireja na morfološkoj razini, a zabilježene su minimalne razlike među njima. Podatci dobiveni istraživanjem upućuju na to da bi hrvatska sibireja trebala dobiti niži taksonomski status u službenoj biljnoj sistematici jer vjerojatno predstavlja samo ekotip altajske sibireje.

Male i izolirane populacije sibireje na visokim hercegovačkim planinama su pred nestajanjem zbog sukcesije flore, odnosno nadiranja šumskih drvenastih vrsta kao što su bukva i munika uz njihove prateće vrste. Zbog toga će očuvanje visokoplaninskih pašnjaka na kojima se javlja sibireja biti moguće jedino kroz aktivno upravljanje (IUCN 1994).

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There are no differences in the restriction pattern for the ITS region in the genomic rDNA indicating that all samples of sibirea belong to the same species as the ITS region proved to be conserved within one taxonomic species. The observed substitutions or inaccuracies in the sequence of the same region gave a difference of 0-2 base pairs between different ITS sequences.

Smaller differences obtained at the molecular level support the hypothesis that sibirea is an old Tertiary relict that shows less variability. That research confirmed Šilić's previous preliminary results, which he personally conveyed to the author (D. Ballian, *Ed. note*), where comparisons of Croatian and Altaic sibireas were made on the morphological level, and minimal differences between them were recorded. The data obtained from the research indicate that Croatian sibirea should receive a lower taxonomic status in the official plant taxonomy because it probably represents only the Altaic sibirea ecotype.

Small and isolated populations of sibirea on the high mountains of Herzegovina are on the verge of disappearing due to the succession of flora, i.e. the encroachment of forest woody species such as beech and mulberry along with their supporting species. Therefore, the preservation of high mountain pastures where sibirea occurs will only be possible through active management (IUCN 1994).

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Ovaj rad u istom obliku nije objavljen niti ponuđen za objavljivanje nekoj drugoj periodičnoj ili neperiodičnoj publikaciji. Autori izjavljuju da nisu u sukobu interesa.

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