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POPULATION GENETIC STRUCTURE OF *SATUREJA SUBSPICATA* BARTL. EX VIS. (LAMIACEAE) IN CENTRAL DINARIC ALPS AND ITS RELEVANCE FOR DNA BARCODING STRATEGIES

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Abstract

Of the four species of the genus *Satureja* (Lamiaceae) that are recognized in Bosnia and Herzegovina, *S. subspicata* has the the widest distribution. It is taxonomically challenging species of geographically limited distribution and little data on its genetic diversity throughout its range is available. We sampled six geographically distinct populations from Bosnia and Herzegovina and applied *nr*DNA (*ITS*1, *ITS*2), chloroplast markers (*mat*K and *trn*L) and AFLP to examine genetic diversity of *S. subspicata* in the center of its distribution range and to explore the possibility of establishing the species DNA barcode. AFLP analysis showed large genetic differentiation among populations as well as moderate correlation between genetic distance among populations and geographic distance among locations. *Mat*K has not proven useful in distinguishing *S. subspicata* from sympatric species. However, *nr*DNA sequences provided necessary resolution power, with *ITS*2 being more informative. Estimates of evolutionary divergence between *nr*DNA sequences obtained in our research and homologous sequences of sympatric *Satureja* deposited in the GenBank reveal closer relationship between geographically proximate populations of different species and slight divergence within *S. subspicata* sequences pool. This outcome highlights the importance of considering overall genetic diversity across the distribution range of a species when assigning DNA barcode.

Key words: AFLP; *mat*K; *nr*DNA; *ITS*1; *ITS*2; DNA barcoding.

Introduction

The genus *Satureja* L., includes aromatic herbs and subshrubs with wide distribution ranging from Mediterranean, including Northern Africa to North-Western China (Govaerts *et al.*, 2003) The genus has been a subject of taxonomic controversy throughout history and over 200 species were described within the genus (Doroszenko, 1986). Over 30 species are distributed in the eastern parts of the Mediterranean area (Silic, 1979; 1984). Recent taxonomic reviews are more moderate with number of species ranging from 38 (Euro+MedPlantBase, 2016) to 50 (Roskov *et al.*, 2016).

Bosnia and Herzegovina (B&H) is located in the center of Balkan Peninsula which is considered one of the most important plant diversity hotspots in European continent (Krystufek & Reed, 2004; Médail & Diadema, 2009). B&H hosts a rich flora; the number of plant species, subspecies, and varieties approaches 4300 (Redzic *et al.*, 2008). Estimated 500 taxa are either endemic or have very narrow distribution. Catalogue of life recognizes 4 distinct species in B&H: *S. montana* L., with wide distribution and *S. cuneifolia* Ten., *S. subspicata*, *S. horvatii* Šilic as species with narrow distribution (Roskov *et al.*, 2016). Also, hybrid *S.*× *karstiana* Justin ex Teyber and *S. hortensis* L. are present in B&H, the latter most likely escaped from cultivation (Silic, 1979).

Among *Satureja* species found in B&H, the most challenging from the taxonomical point of view is *S. subspicata*. Catalogue of life (Roskov *et al.*, 2016) lists four species with 12 subspecific, varietal and form ranks as synonyms for *S. subspicata* (*S. illyrica* Host, *S. montana* var. *blavii* Asch. ex K. Malý, *S. montana* var. *illyrica* (Host) Benth., *S. montana* subsp. *illyrica* (Host) Nyman, *S. pygmaea* Sieber ex Vis., *S. pygmaea* var. *obcordata* Horák, *S. subspicata* var. *blavii* (Asch. ex K. Malý) K. Malý, *S. subspicata* var. *macedonica* Velen., *S. subspicata* var. *obcordata* (Horák) Silic, *S. subspicata* f. *obovata*Silic, *S. subspicata* f. *scabrifolia* (Rohlena) Silic, *S. subspicata* var. *scabrifolia* Rohlena).

However, EUNIS (European Nature Information System) maintained by European Environmental Agency recognizes *S. subspicata* as a synonym of *S. montana* ssp. *illyrica* Nyman. Considering the importance of EUNIS as a base for implementing the EU and global biodiversity strategies as well as the general distribution of *S. subspicata* in Bosnia and Herzegovina, we decided to explore genetic variation of this species in the Central Balkans and possibly, to determine its unique DNA barcode.

S. subspicata is known as mountain savory. It represents a typical Illyric-Balcanic floristic element with wide distribution ranging from Italy near Trieste, across Slovenian part of Istrian Peninsula (south Notranjska), Bosnia and Herzegovina, Croatia and Montenegro to northern Albania (Soljan *et al.*, 2009). Compared to other Balkan endemic savory species, it has the largest distribution (Silic, 1979). Most frequently, it is found at higher altitudes at crests and peeks exposed to winds, in shallow soils with limestone and dolomite as bedrock. These habitats primarily belong to endemic plant associations within the endemic Dinaric alliances *Chrysopogoni-Satureionsubspicatae* H-at & H-ić and *Scorzonerion villosae* H-ić. Its vertical distribution ranges between 200 and 1800 m alt (Silic, 1979). It often co-occurs with *S. montana* at the same habitats, and sometimes with *S. cuneifolia* and *S. horvatii*. *S. subspicata* is classified as LC in Red List of FB&H (Dug *et al.*, 2013), however, unsustainable exploitation puts considerable pressure on wild populations.

Literature review revealed surprisingly little data on genetic diversity of *S. subspicata* throughout its range. Bezic *et al.,* (2009) analyzed internal transcribed spacer (*ITS*) sequence variability in specimens collected in Littoral Dinaric Belt. Considering the lack of genetic data and heterogeneity of ecological conditions in terms of spatial distribution and geomorphology of *S. subspicata* area we combined nuclear (*ITS1*, *ITS2*), chloroplast markers (*mat*K and *trn*L) and Amplified Fragment Length Polymorphism (AFLP) fingerprinting to: (1) examine genetic diversity of *Satureja subspicata* in Bosnia and Herzegovina; (2) examine the relation between the genetic and geographic distribution of the sampled populations and (3) to evaluate the applicability of the applied markers for barcoding purposes.

Material and Methods

Collection of samples: Plant material of *Satureja subspicata* was collected from six spatially distinct populations in Bosnia and Herzegovina during August and September 2015. Precise geographic locations are listed in Table 1. Voucher specimens were deposited in the National Museum of Bosnia and Herzegovina [Voucher no. (SARA): 0051544, (SARA): 0051545]. Sampled individuals were spatially separated within the populations. Leaf material was silica-dried and stored until the analysis.

***DNA* extraction, amplification and sequencing of mat*K* and *ITS*1and *ITS*2:** Silica-dried and pulverized leaves (cca. 200 mg) were used for DNA extraction procedure. Total genomic DNA was extracted using hexadecyltrimethyl ammoniumbromide (CTAB) based protocol described by Padmalatha *et al*., (2008). Genomic DNA was analyzed by electrophoresis in SB (Sodium borate) buffer in 1,5 % (w/v) agarose gel and visualized under UV light after staining with Midori green (Nippon Genetics Europe).

A fragment of *maturase* K gene was amplified using matK_F_BHEPS (5'-CGATCTATTCATTCAATA-3') and matK R BHEPS (5'-TCTAGCACACGAAAGTCGA-3') primers according to Sun *et al*., (2001). Amplification was performed in 30 µl reactions consisting of 1 µl of template DNA, 2 mM Tris-HCl (pH 8.0), 10 mM KCl, 0.2 µM of each primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$ and 1 unit of Taq DNA polymerase (Gdansk). Cycling parameters were 94ºC for 1 min, 48ºC for 30 s, 72ºC for 1 min, 72ºC for 7 min with the total of 26 cycles. *ITS1* and *ITS2* fragments were amplified in separate reactions using primers described by White *et al.,* (1990). PCR reactions were performed under the same chemical regime as for the *mat*K. PCR amplification was carried out in 30 cycles (95ºC for 3 min, 95ºC for 30 s, 50ºC for 30s, 72ºC for 1 min, 72ºC 10 min).

PCR products were sequenced by Macrogen Inc. Europe as part of their regular capillary DNA sequencing services. *Mat*K amplicons were sequenced in both directions while *ITS1* and *ITS2* fragments were sequenced in one direction.

***PCR-RFLP* method:** Plastidial *trn*L (UAA) intron was amplified using the primer pair c and d (Taberlet *et al.*, 1991). PCR reactions were performed in total volume of 15 µl containing 1 µl of template DNA, 2 mM Tris-HCl (pH 8.0), 10 mM KCl, 0.2 µM of each primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$ and 1 unit of Taq DNA polymerase (Gdansk). PCR program consisted of 30 cycles (94°C for 45 s, 51°C for 30 s, 72°C for 45 s, 72°C 10 min) in ABI GeneAmp® PCR System 9700. Amplicons were analyzed by electrophoresis in 1.5 % (w/v) agarose gel prepared in SB buffer and visualized under UV light after staining with Midori green (Nippon Genetics Europe). Successfully amplified fragments were digested with enzymes: *Taq*I, *Hpy*F31, *Hinf*I, *Hind*III, *Hind*II, *Rsa*I, *Eco*RI, *Bam*HI, *Alu*I, *Ava*II and *Bsa*I (BioLabs New England) in individual reactions. Digestion was performed according to the manufacturers' instructions. Fragments separation was performed in 2 % agarose gel against 50 bp DNA Ladder (BioLabs New England).

***AFLP* method:** AFLP analysis was performed according to the general principles set forth by Vos *et al*., (1995) with modifications allowing for capillary analysis. Protocol optimization was performed following the guidelines by Trybush *et al*., (2006). Total of 59 individuals, 10 from each population, were analyzed with three primer pair combinations. Digestion, ligation of the adapters and pre-selective amplification were performed according to Trybush *et al*., (2006).

Selective PCR amplification was performed in individual reactions. The reaction volume was 10 µl which included 1 µl of 30-fold diluted preselective PCR product, one fluorescently labeled selective PCR primer [*Eco*RI (6-FAM)-A-GAC, *Eco*RI (TAMRA)-A-AGC or *Eco*RI (HEX)-A-TCC] at concentration 0.7 µM, an unlabeled primer *Mse*I-C-AC 0.7 µM, TaqNovaHS reaction buffer 1X (Gdansk), dNTPs 0.2 mM, $MgCl_2$ 1.5 mM and TaqNovaHS DNA Polymerase 1.5 U/µl (Gdansk). Selective amplification was performed in 36 cycles according to the following thermal protocol: denaturation at 94ºC for 30 s, annealing at 65ºC for 30 s with 0.7 ºC decrease per cycle for the following 12 cycles and 56ºC for the remaining 23 cycles, extension at 72ºC for 1 min. Amplified PCR products were mixed with Size Standard LIZ 500 (GeneScan) and formamide and analyzed on Applied Biosystems 3500 Genetic Analyzer. Electropherograms were analyzed using GeneMapper 5, followed by manual adjustment. The minimum relative intensity for peak detection threshold was 200 rfu. The range for allele call was 100 – 500 bp. The reproducibility was tested using 15 randomly selected samples and calculated as the ratio of number of fragment differences / total number of observations (Bonin *et al.*, 2004).

Plastidial mat*K* and nr*DNA* sequence assembly and annotation: Plastidial *mat*K and *nr*DNA sequence identification analysis from *S. subspicata* were performed against the FASTA program (Pearson, 1994). BLAST network service (Benson *et al.*, 2003) in GenBank at NCBI was used for final sequence identification, searching for the best identity and similarity scores in local databases. Sequencing reads were assembled using DNASTAR's Lasergene software EditSeq (Burland, 2000) and examined manually by electropherograms for sequencing errors. Multiple sequence alignment (MSA) analysis for two *mat*K and two concatenated *nr*DNA sequences (*18S* RNA- *ITS1-5.8S* RNA and *5.8S* RNA-*ITS2*) were performed using ClustalW Ver.1.6 (Thompson *et al.*, 2011) under default parameters, respectively. MSA analyzed sequences and outputs were optimized using Jalview 2.9.0b2 (Waterhouse *et al.*, 2009) and edited by Bioedit v5.09 (Hall, 1999).

Table 1. Geographical origin of the analyzed populations of *S. subspicata*.

No. [a]	Locality	Longitude (E)	Latitude (N)	Altitude (m)
1.	Bijela Gora	18°33'29"	42°37'29"	1270
2.	Klinja	18°34'06"	43°10'52"	1090
3.	Slovinj	16°57'22"	43°59'10"	1380
4.	Doljanka valley, Mt. Plasa	17°41'08"	43°41'08"	460
5.	Bosiljna, Mt Čvrsnica	17°29'27"	43°30'09"	1319
6.	Višegrad	19°12'21"	43°44'21"	355

[a] – Numbers correspond to the markings in Fig. 1.

Sequence alignment and distance analysis: DNA sequences obtained in this study were subjected to similarity search against nucleotide sequence database using the NCBI (National Centre for Biotechnology Information) BLASTN service (Benson *et al.*, 2003; Zhang *et al.*, 2000). Sequence alignments, estimates of evolutionary divergence between sequences and construction of Maximum Likelihood tree were performed using MEGA6 (Molecular Evolutionary Genetics Analysis) software (Tamura, 1992; Tamura *et al.*, 2013). Default parameters were applied.

***AFLP* data analysis:** AFLP bands were scored for presence (1) and absence (0) and the binary data set representing combined AFLP profile of each sample was created. Total and within-population genetic diversity was examined using Nei's statistics (Nei, 1987) and Shannon's information index of genetic diversity. Degree of population differentiation (Fst) was calculated as (Ht-Hs)/Ht. Analysis of molecular variance (AMOVA) was performed in order to clarify hierarchical apportioning genetic variance (Exoffier, 1992).

Genetic distance between populations was estimated according to Nei (1987), since pairwise matrices were generated by Jaccard's coefficient of similarity (Jaccard, 1908). The Neighbor-Joining cluster analysis (Saitou & Nei, 1987) was performed based on Nei's genetic distance. Genetic relationships among individual and populations were assessed via principal coordinates analysis (PCO) based on Jaccard's coefficient of similarity. Correlation between the matrices of genetic and geographic distance as well as between similarity indices and geographic distances were estimated using Mantel matrix correspondence test (Mantel, 1967).

In order to examine population structure and assign AFLP individual profile to a specific population, we used the Bayesian model based clustering procedure within Structure version 2.3.4. (Pritchard, 2000). A burn-in period of 200,000 and 500,000 iterations for data collection was applied. Structure harvester ver. 0.6.94. application (Earl & vonHoldt, 2012) was used to estimate the most probable K value for the analyzed data (Evanno *et al.*, 2005).

Nei's genetic statistics, Shannon's information index, AMOVA, geographic distance estimation and Mantel matrix correspondence test were carried out in GenAlEx version 6.5 (Peakall & Smouse, 2012). Jaccard's coefficient of similarity and principal coordinates analysis (PCoA) were calculated using PAST version 2.17 (Hammer *et al.*, 2001). Construction of Neighbor-joining dendrogram was performed using MEGA 7 (Molecular Evolutionary Genetics Analysis) software (Tamura *et al.*, 2004; Kumar *et al.*, 2016).

Results

***cp*DNA:** High quality DNA was successfully isolated from all 105 samples. The *trn*L intron was amplified in all analyzed samples. The amplicon size was 550 bp, which corresponds to the size expected, based on *in silico* PCR using *S. hortensis* AY506611 as a template. Of the 11 restriction enzymes applied, only three found recognition site within the amplicon: *Hinf*I, *Alu*I and *Hae*III. Upon the analysis of the generated restriction patterns no polymorphism was detected i.e. all the individuals exhibited the same haplotype. Consequently, no intrapopulation or interpopulation polymorphism was detected. Both haplotype distance and intrapopulation haplotype diversity equal 0.

Partial *mat*K sequence of 878 bp was obtained and deposited in the GenBank database (GenBankID: KX954591). Since sequencing was performed in both directions we were able to obtain confident DNA sequence.

***nr*DNA:** Two concatenated *nr*DNA consensus sequences (*8S* RNA- *ITS1-5.8S* RNA (277 bp) and *5.8S* RNA-*ITS2* (296 bp)) were obtained and deposited in the GenBank database (GenBankID's: KY380095, KY380100).

***AFLP* data:** Reproducibility test showed 7.7% inconsistencies. Following the guidelines by Bonin *et al.,* (2004) we increased minimum relative intensity peek threshold to 200 rfu as most of the inconsistencies were found among low intensity peeks. Using three primer-pair combinations we scored 511 polymorphic markers in a set of 59 specimens. The genetic diversity parameters of the observed populations are summarized in Table 2. Unbiased diversity and Shannon's information index of observed populations ranged from 0.128 (Slovinj) to 0.235 (Bijela Gora) and from 0.177 (Slovinj) to 0.321 (Bijela Gora), respectively. Mean value of unbiased genetic diversity for all populations was 0.186, while for Shannon's information index was 0.256.

Overall Fst value was 0.262 which indicates large genetic differentiation among the observed populations. This result was confirmed by the analysis of molecular variance (Table 3). AMOVA showed no regional stratification (0%), since 29% (P=0.001) of genetic variance was among six observed populations of *S. subspicata*. Portion of genetic variance within populations was 71%.

Table 2. Genetic diversity within six populations of *S. subspicata*.

Population	N	I	uH
Bijela Gora	10	0.321	0.235
Slovinj	10	0.177	0.128
Doljani	10	0.241	0.176
Bosiljna	10	0.262	0.189
Visegrad	10	0.290	0.211
Klinje	9	0.244	0.179
Total (mean)	**-**	**0.256**	**0.186**

I – Shannon's information index; uH – unbiased diversity

Analyses of Nei's genetic distance revealed the lowest value between Bosiljna and Doljani locations (0.057), while the highest value was noticed between Slovinj and Visegrad locations (0.178) (Table 4). Correlation between matrices of Nei's genetic distance among populations and geographic distance among locations showed moderate value (r=0.616, P=0.02), indicating that the two distances had a positive correlation (Fig. 2). Also, the result of Mantel test between Jaccard's coefficient of similarity and geographic distance matrices revealed negative moderate but statistically significant value (r=-0.628, P=0.01), showing that larger geographic distance between sites follows lower similarity indices (Fig. 3).

Neighbor-Joining cluster analysis based on Nei's genetic distance clearly showed separate position of Višegrad population (Fig. 4). The second cluster makes Slovinj and Doljani, and third Klinje and Bosiljna accompanied by Bijela Gora. Principal coordinates analysis (PCoA) was estimated based on Jaccard's coefficient of similarity (Fig. 5). On the whole, 31 % of total variance is represented: 12.9% by the first, 11% by the second and 7.1% by the third component. PCoA plot clearly shows separation of Slovinj population from other five populations (Višegrad, Bijela Gora, Bosiljna, Doljani and Klinje). The same population has reduced genetic variation. Višegrad shows tendency of separation from other populations, since clear differentiation between Bijela Gora, Bosiljna, Doljani and Klinje was not established.

Subsequent ΔK analyses based on range K=1-10 with 10 iterations, revealed that K = 2 was the most probable one (Fig. 6). According to Structure analyses, samples from five populations (Bijela Gora, Klinje, Slovinj, Doljani and Bosiljna) were assigned to the first cluster (>90%), while Višegrad population was assigned to the second cluster (>99%) (Fig. 7). Distribution of samples from Bijela Gora showed that although 90% were assigned to the first cluster, 10% percent samples were also assigned to second cluster. Percentage of samples of Klinje, Slovinj, Doljani and Bosiljna assigned to the first cluster was >96%.

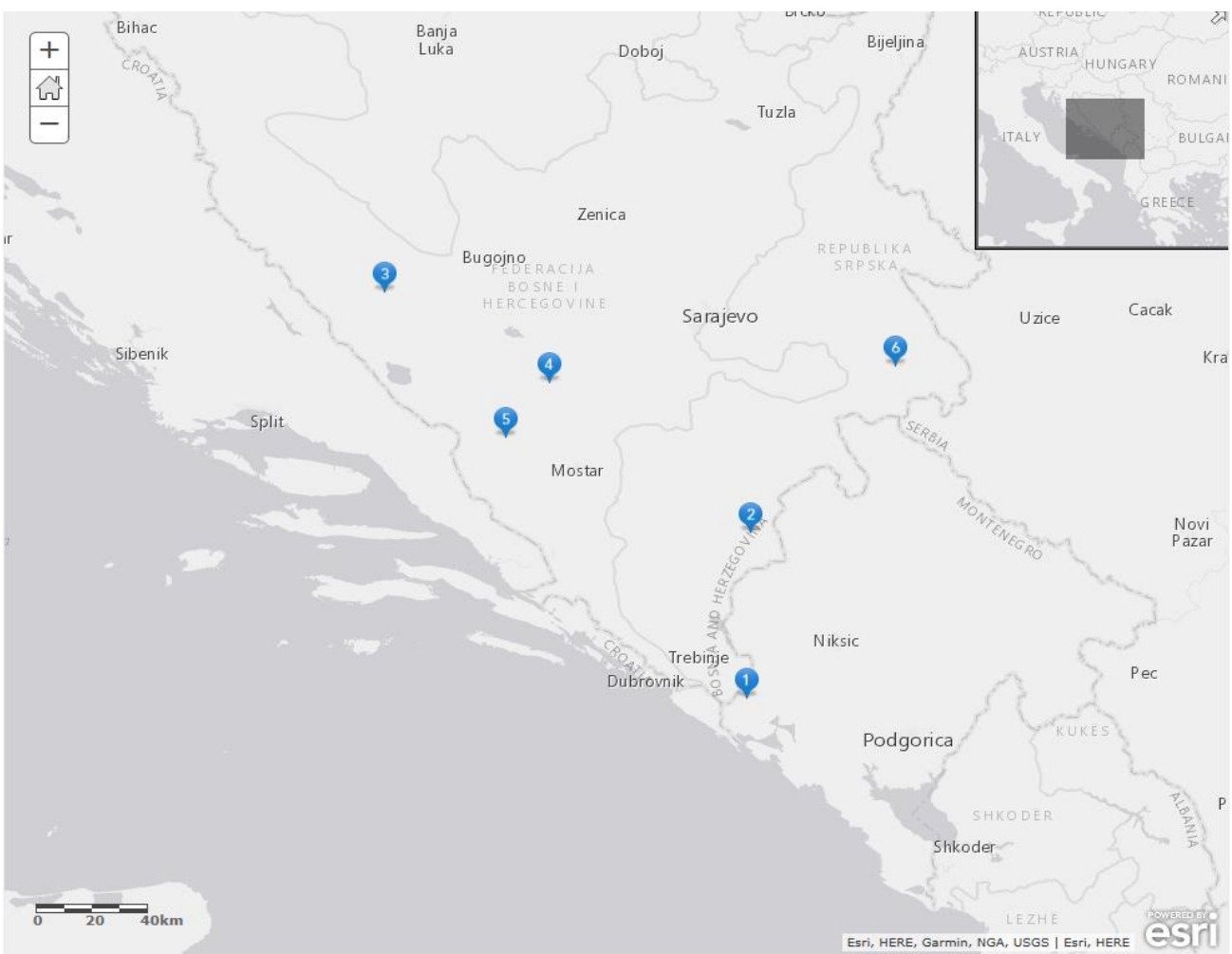

Fig. 1. Geographic position of the sampled populations within Bosnia and Herzegovina (populations are numbered according to Table 1).

Table 3. Analysis of molecular variance (AMOVA) summary for *S. subspicata*

Source of variation	df	SS	MS	Est. Var.	%	Fst	p value
Among Regions	2	485.507	242.754	0.189	0.00		
Among Pops	3	717.185	239.062	19.483	0.29	0.290	0.001
Within Pops	53	2525.544	47.652	47.652	0.71		
Total	58	3728.237		67.324	1.00		

DF - degree of freedom; SS - sum of square; MS - mean squares

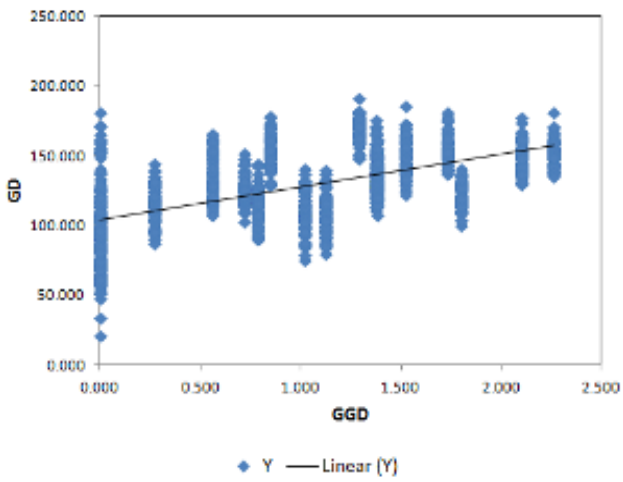

Fig. 2. Plot of Mantel test between Nei's genetic distance among six populations and geographic distance.

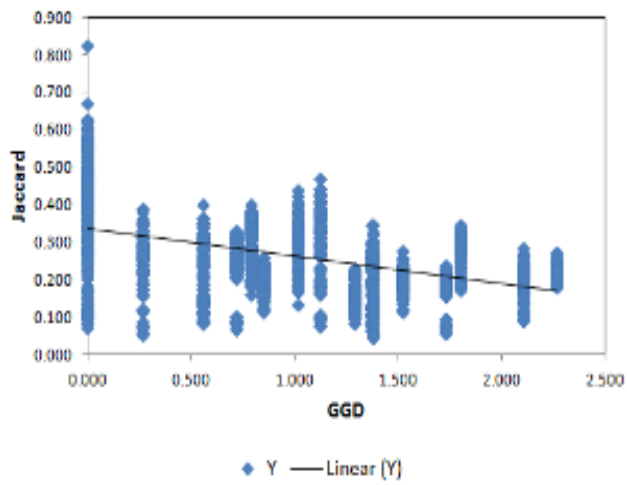

Fig. 3. Plot of Mantel test between Jaccard's coefficient of similarity and geographic distance.

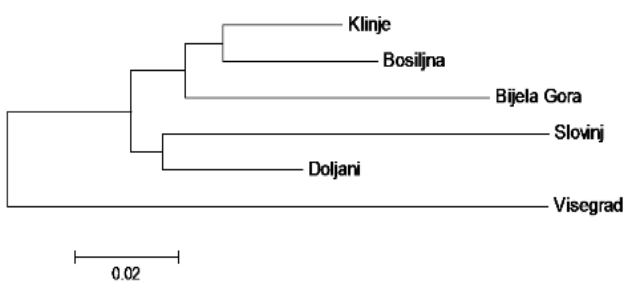

Fig. 4. Neighbor-Joining dendrogram based on Nei's genetic distance among six populations of *S. subspicata*.

Table 4. Nei's genetic distance among six populations of *S. subspicata*.

	BG	SL	DO	BO	VI	KL
BG	0.000					
SL	0.164	0.000				
DO	0.106	0.102	0.000			
BO	0.095	0.128	0.079	0.000		
VI	0.170	0.178	0.144	0.157	0.000	
KL	0.093	0.125	0.073	0.057	0.157	0.000

Legend: BG= Bijela Gora; SL=Slovinj; DO=Doljani; BO=Bosiljna; VI=Visegrad; KL=Klinje

Table 5. Estimates of evolutionary divergence between *mat*K sequences of four *Satureja*speciesfound in Central Dinaric Alps.

S. subspicata (KX954591*)			
S. horvatii (KX954592)	0		
S. montana (AY840167)	0	0	
S. cuneifolia (GU381777)	0.001	0.001	0.001

**mat*K sequence obtained in this research

Discussion

Intra and interpopulational diversity of *S. subspicata*: PCR-RFLP of *trn*L intron failed to reveal any polymorphism within the analyzed sample with the used restriction enzymes. Given that Taberlet *et al*., (2007), following the analysis of *trn*L DNA sequences in 11404 plant species, estimated intraspecific variability of the intron sequence between 5.9 and 55.0 %, our results are justifiable. PCR-RFLP explored a limited subset of nucleotide positions within *trn*L that were recognized by the used restriction enzymes. Clearly, *trn*L is not the best choice when characterizing diversity within single plant species.

AFLP markers revealed marked genetic differentiation among the studied populations of *S. subspicata* (Fst of 0.262). Interpopulational variance shown by AMOVA (29% variance distributed among populations with 71% of within-population variance (P=0.001); Table 3) appears to be extreme; however, the nature of AFLP markers should be taken into consideration. AFLP are fast evolving markers with probable homoplasy (Bonin *et al.*, 2007) and they generally tend to overestimate interpopulational variance. Also, they provide better resolution for closely related populations. In a study by Lopez-Sepulveda *et al*., (2013) AFLP markers presented larger genetic variation among populations in comparison with microsatellites (10.4% vs. 4.5% and 16.4% vs. 8.5%) for the same set of populations of endemic *Myrceugenia* species.

Two populations stand out with respect to intrapopulational diversity: Slovinj with the lowest unbiased diversity of 0.128 and Bijela Gora with the greatest unbiased diversity of 0.235. PCO plot (Fig. 5) also shows separation and tight grouping of Slovinj population. Like other *Lamiaceae*, *Satureja* are protandrous species which should assume high level of outcrossing. However, efficacy of protandry as an isolating mechanism has been questioned. During anthesis, the flowering phases among flowers of the same individual overlap enabling geitonogamy to various degrees (Navarro, 1997; Classen-Bockhoff *et al.*, 2003). There emerges physiological self-incompatibility as an obstacle; however Navarro (1997) raises an option of partial self-incompatibility as an alternative in protandrous species. Slovinj population is located at 1380 m alt at the northern border of *S. subspicata* distribution range. The population is exposed to heavy wind throughout the flowering season which presents heavy burden for insect pollinators. In such circumstances, geitonogamy may account for low unbiased diversity observed in this population.

In Bijela Gora, *S. subspicata* is sympatric to *S. horvatii*, *S. montana* and *S. cuneifolia* which creates potential hybridization zone. Although specimens with doubtful phenotype were avoided during sampling, possibility of interspecific introgression must be allowed.

Relation between genetic and geographic distribution of the sampled populations: Mantel correlation test revealed moderate but statistically significant correlation between genetic and geographical distances (r=0.616) which indicates that isolation-by-distance exists. The lowest value of genetic distance was found between the two geographically closest populations of Bosiljna and Doljani (0.057). These two populations are located at the southern or northern borders of Mt. Cvrsnica. However, these two populations significantly differ in altitude (859 m) and phenological isolation is expected (Table 1, Fig. 1). Doljani population is located in the valley, in the Canyon of Doljanka River, under the ridges of Mt. Cvrsnica. It is a typical habitat for *S. subspicata*. Thus, it is plausible that the founder population to Doljani is actually located much higher, at the ridge of the canyon and therefore much closer to Bosiljna population. In addition to being the most geographically distant populations, Slovinj and Višegrad are located at the periphery of the distribution range so the marked genetic distance is understandable. The result of correlation between genetic and geographical distances is concordant with the result of Mantel test between Jaccard's coefficient of similarity and geographic distance.

The population genetic structure analyses (NJ cluster analysis and STRUCTURE) clearly distinguish two well supported clusters with five populations assigned to one cluster and Višegrad population alone making one cluster. Višegrad is an interesting population insofar as it is located outside of the distribution range of *S. subspicata* in the Balkans, while the other populations are located within or at the border of the distribution range. Such isolated populations are expected to diverge via effects of spatial isolation in relation to central populations, genetic drift and natural selection (Lesica & Allendorf, 1995). Interestingly though, the Structure plot (Fig. 7) also singles our Bijela Gora population with the largest number of samples not assigned to the second cluster (10%). This may be a reflection of anticipated interspecific introgression from sympatric populations of *S. horvatii*, *S. montana* and *S. cuneifolia*. Confirmation of this hypothesis requires further investigation.

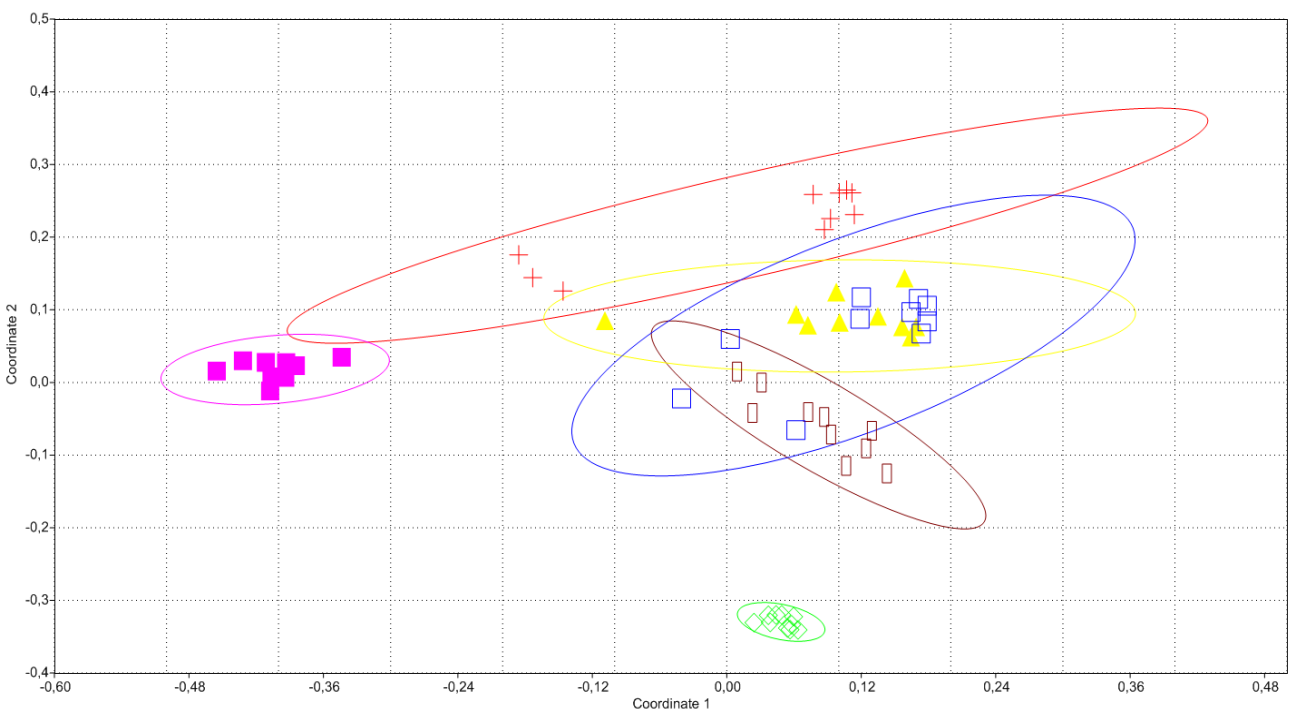

Fig. 5. Principal coordinate analysis (PCoA) plot for samples from six observed populations (+ = Bijela Gora; ◇ =Slovinj; ▯=Doljani; ▲ =Bosiljna; ■ =Višegrad; □ =Klinje)

Applicability of *mat*K and *nrDNA* for *DNA* barcoding purposes: The goal of DNA barcoding is to find one or a few regions of DNA that will distinguish among the majority of the world's species. Various DNA sequences were proposed for land plants. CBOL Plant Working Group (Hollingsworth *et al.*, 2009) proposed 2-locus combination of *rbc*L+*mat*K as the core plant barcode. However, comprehensive research showed that *ITS* sequence may be necessary to provide better resolution at species level (China Plant BOL Group –Li *et al.*, 2011; Hollingsworth, 2011; Hollingsworth *et al.*, 2011; Zahra *et al*., 2016). Hollingsworth (2011) reviewed major challenges for the use of *nr*DNA as a barcode and argues that even when the amplification and sequencing of template proves difficult, truncated sequence (*ITS*2) still provides gain in discriminatory power.

Yu *et al*., (2011) evaluated the entire *mat*K gene to find a region of 600-800 bp that is highly variable, avoids mononucleotide repeats and with priming sites conservative enough to design universal primers. The authors proposed *mat*K fragment of approximately 776 bp based on the inclusion of the most variable sites, best representation of the entire *mat*K gene as well as good sequence quality. This fragment contains sequence from 472 to 1248 bp of the whole *mat*K gene sequence of *Arabidopsis thaliana*. The *mat*K sequence of *S. subspicata* obtained in our research corresponds to 438-1292 position of *mat*K gene mapped against *A. thaliana* reference chloroplast genome (NC_000932.1; matK position 2056-3570). It evidently corresponds to the criteria for DNA barcoding using *mat*K determined by Yu *et al.*, (2011). Therefore, m*at*K sequence of *S. subspicata* of 878 bp was submitted and mined in BOLD Systems v3 Database (BOLDID's: BHEPS002-16).

However, BLASTN similarity revealed low discriminatory power of this region within *Lamiaceae*. Sequences with ≥ 99% identity (> 98% sequence coverage) were found in genera *Satureja*, *Clinopodium*, *Mentha* and *Monarda*. In order to compare our sequence with the sequences of *Satureja* species that co-occur with *S. subspicata* in Central Dinaric Alps we retrieved *mat*K sequences of *S. subspicata*, *S. horvatii*, *S. montana* and *S. cuneifolia* available in GenBank. The results of evolutionary divergence estimates clearly indicate that the sequenced 878 bp fragment of *mat*K cannot confidently distinguish among the analyzed species (Table 5).

The two concatenated *nr*DNA consensus sequences (*8S* RNA- *ITS1-5.8S* RNA and *5.8S* RNA-ITS2) of *S. subspicata* that were obtained in our research were analyzed for discriminatory power against co-occurring *Satureja* species in the Central Dinaric Alps. *S. hortensis* (AY227143) served as an out-group. Despite cosmopolitan distribution of *S. hortensis* and possible sympatry with the species of interest (Roskov *et al.*, 2016), the origin of this particular sequence is Macaronesian Islands (Trusty *et al.*, 2004) so spatial separation can be safely assumed. *ITS*1 and *ITS*2 DNA sequences from sympatric species were retrieved from GenBank. Although only 19 bp longer, *ITS*2 evidently provides better resolution (Tables 6 and 7). *S. visianii* is endemic to Peljesac peninsula. Even though its distribution range does not extend into Central Dinaric Alps it was included in the analysis because the population whence one of *S. subspicata* sequences originates (EU823288) belongs to Littoral Dinaric Belt. Evidently, sequences obtained in our research clearly separate *S. subspicata* from the other species, most notably from *S. montana*, which supports two distinct species viewpoint. However, *S. subspicata* sequence retrieved from GenBank (EU823288), shows low divergence from our sequence, but it also shows high similarity to *S. visiani* sequence from the same region.

Molecular Phylogenetic analysis by Maximum Likelihood method based on a matrix of pairwise distances using Maximum Composite Likelihood approach also supports *ITS*2 as a marker with better discriminatory power (Figs. 8 and 9). Also, both *ITS* markers indicate close relationship between *S. subspicata* and sympatric *Satureja* species with narrow distribution (*S. horvatii* in Central Dinaric Alps and *S. visianii* in Litoral Dinaric Belt).

Table 6. Estimates of evolutionary divergence between *ITS*1 sequences of six *Satureja* species.

S. cuneifolia (EU823290)								
S. horvatii (KY380094)	0.015							
S. hortensis (AY227143)	0.060	0.063						
S. montana (EU823287)	0.004	0.019	0.063					
S. montana (JQ669135)	0.015	0.015	0.060	0.019				
S. montana (GU381442)	0.007	0.007	0.056	0.011	0.007			
S. subspicata (EU823288)	0.007	0.007	0.056	0.011	0.007	0.000		
S. subspicata (KY380093*)	0.015	0.000	0.063	0.019	0.015	0.007	0.007	
S. visianii (EU823289)	0.007	0.007	0.056	0.011	0.007	0.000	0.000	0.007

**ITS*1 sequence obtained in this research

Table 7. Estimates of evolutionary divergence between *ITS*2 sequences of six *Satureja* species.

S. montana (JQ669135)								
S. montana (GU381442)	0.004							
S. cuneifolia (EU823290)	0.007	0.011						
S. visianii (EU823289)	0.014	0.018	0.014					
S. montana (EU823287)	0.025	0.028	0.025	0.032				
S. subspicata (EU823288)	0.007	0.011	0.007	0.007	0.025			
S. hortensis (AY227143)	0.032	0.032	0.032	0.032	0.049	0.025		
S. subspicata (KY380098*)	0.007	0.011	0.007	0.014	0.025	0.007	0.032	
S. horvatii (KY380099)	0.007	0.011	0.007	0.014	0.025	0.007	0.032	0.000

**ITS*2 sequence obtained in this research

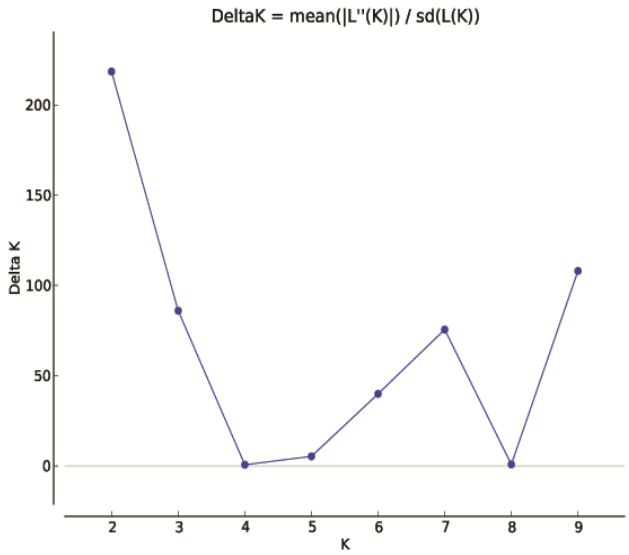

Fig. 6. Plot of the most probable ΔK values from the Structure analyses of 59 samples and six populations of *S. subspicata*, obtained through Structure harvester ver. 0.694 application.

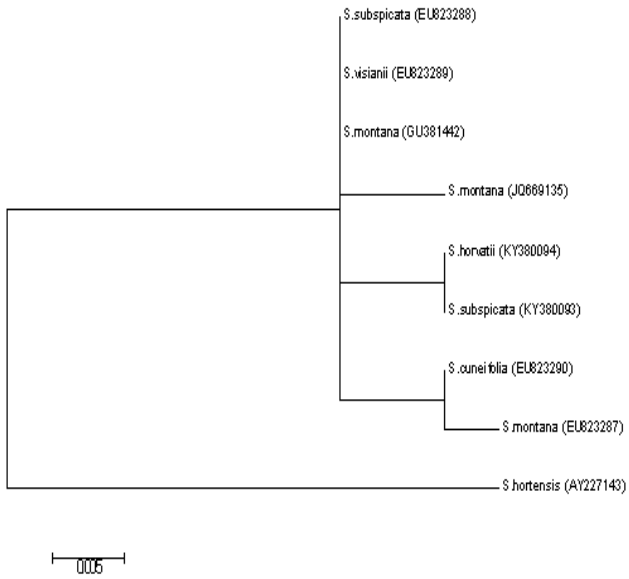

Fig. 8. Phylogenetic relationship of *Satureja* species based on *ITS*1 sequences.

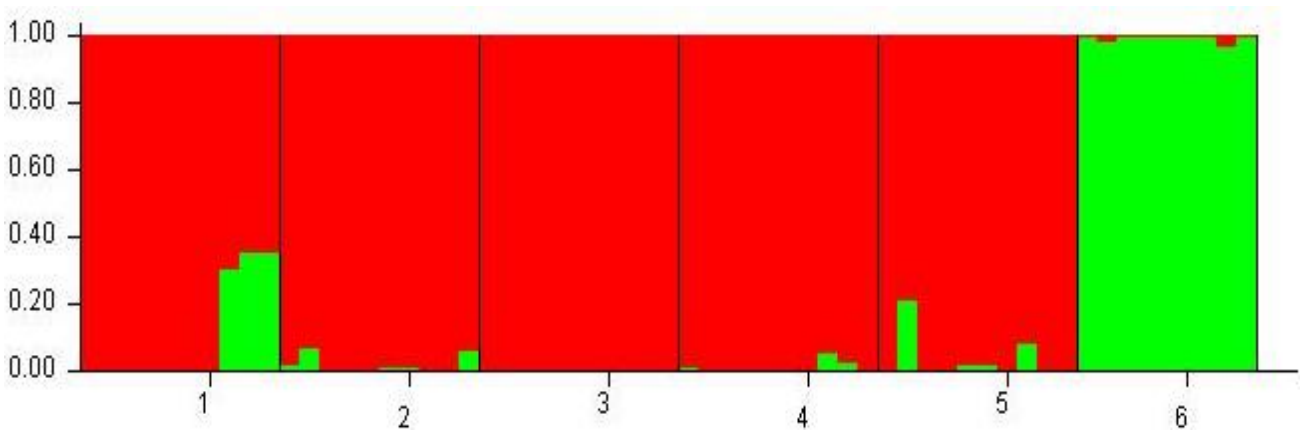

Fig. 7. Structure plot of 59 samples within six populations of *S. subspicata* assigned to two clusters (K=2) (1=Bijela Gora; 2=Klinje; 3=Slovinj; 4=Doljani; 5=Bosiljna; 6=Višegrad).

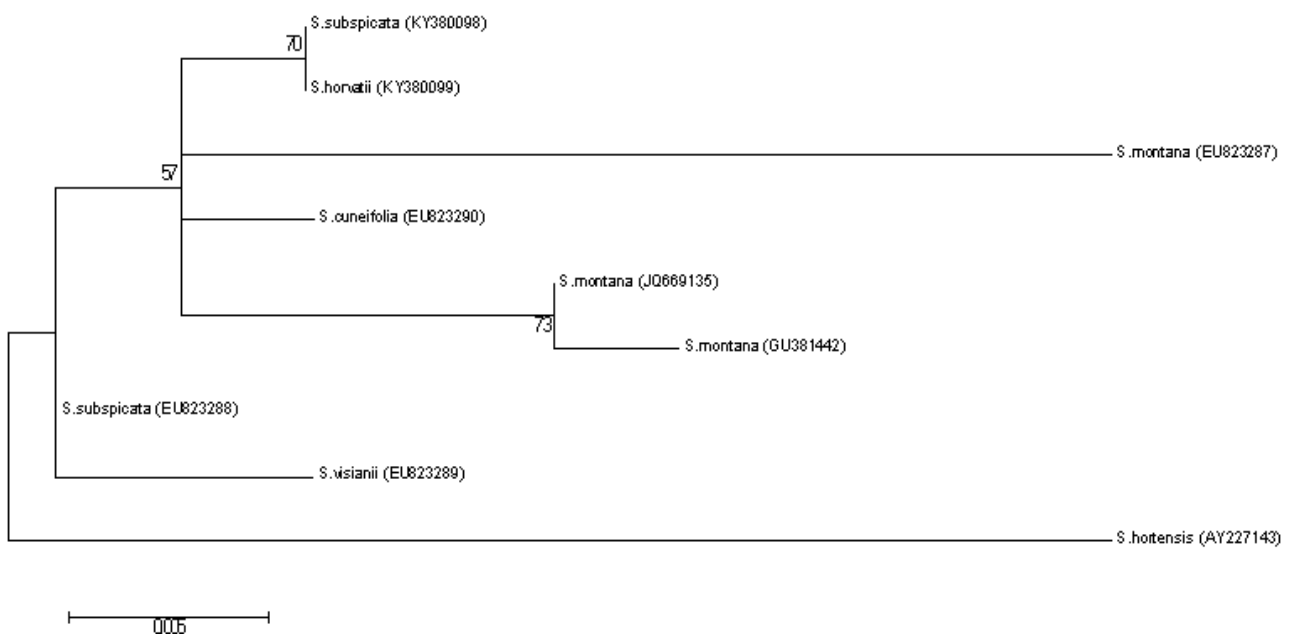

Fig. 9. Phylogenetic relationship of *Satureja*species based on *ITS*2 sequences.

Conclusions

Although PCR-RFLP of *trn*L intron failed to detect any polymorphisms, AFLP analysis revealed marked genetic differentiation among the studied populations of *S. subspicata* from Central Dinaric Alps. Portion of variance distributed among the populations is well within the expected range considering the markers used as well as geographical distribution of the populations. *Mat*K has not proven suitable DNA marker for DNA barcoding of *Satureja* species. Although *nr*DNA sequences provide better resolution, information on the origin of the sequenced specimen must be carefully considered in determining the value of particular sequence as a DNA barcode. Also, examination of overall genetic diversity across the distribution range of the species should be prerogative when assigning DNA barcode.

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