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Juniperus excelsa s. str. in Crimea – differentiation and history inferred from genetic and morphological markers

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ABSTRACT

Juniperus excelsa s. str. (Greek juniper) in Crimea is a relic species on the limits of its range, and represents the Mediterranean flora in the Sub-Mediterranean part of the peninsula. Its origin and history in this area remains unresolved. We aimed to analyze phylogeny and potential demographic expansion of the juniper in the Crimea as well as to study its morphological differentiation. We analyzed plant material from 59 trees inhabiting eight populations. Genetic variation assessments were based on the four non-coding chloroplast DNA (cpDNA) fragments and the nuclear internal transcribed spacer region ITS1-5,8S-ITS2 (ITS). To examine the morphological differentiation, eight measured/counted traits of cones, seeds, and shoots were chosen and eight ratios were calculated. Morphological parameters were compared using ANOVA, Student's *t* test, discrimination analysis and Kruskal-Wallis and U Mann-Whitney tests. Two cpDNA fragments were polymorphic and, in total, 10 cpDNA haplotypes were found. Haplotype diversity (H_d) ranged from 0.0 to 0.9. Based on both cpDNA and ITS sequences variation, phylogenetic analyses revealed a close relationship of the Crimean junipers to the individuals from other parts of the species range. In general, our molecular results confirmed the low level of genetic differentiation of *J. excelsa* individuals inhabiting different parts of the species range, likely resulting from a common ancestry. Only slight morphological differences were found between populations with different geographic location or habitat. The analyzes showed the distinctness of the populations from the southern part of the coast. Some unique morphological and molecular features of southern coastal populations imply that they are remnants of Late Pleistocene abundant forests. We suggest that the recent fragmentation of the *Juniperus* populations in the Crimean Peninsula could have arisen during the Atlantic period of the Holocene.

KEY WORDS

biometry, cpDNA, Crimean Sub-Mediterranean, isolated populations, juniper, ITS

INTRODUCTION

Juniperus excelsa M.Bieb. (Greek juniper) is a Mediterranean species, and in Crimea it can be found only in the narrow area located south of the Crimean Mountains, referred to as sub-Mediterranean (Cordova 2016). Within its distribution range, the species is variable and its taxonomy is not fully resolved. It is regarded as a complex species, including var. *excelsa* and var. *polycarpus* (Farjon 2005), and the relation of this taxon with *J. turcomanica* and *J. seravshanica* (Farjon 2005; Adams 2014) is a subject of debate. *Juniperus turcomanica* and *J. seravshanica* sometimes are even treated as varieties of *J. excelsa* s. l. (Adams 2016). In Crimea, only *J. excelsa* s. str., or *J. excelsa* var. *excelsa*, can be found. Although this is one of the two northernmost positions of the species (Farjon 2005; Cordova 2007; Adams 2014), the first mention and morphological description of *J. excelsa* (as *J. lycia*) stems from Crimea, in 1789, reported by Pallas (Farjon 2005; Adams 2014). *Juniperus excelsa* is a tree-like juniper, a light demanding pioneer species, highly resistant to severe drought, cold conditions, and shallow, degraded soils (Magyari et al. 2008; Ozkan et al. 2010). It is a major mountain forest element in the East Mediterranean Basin and sub-Mediterranean region. The species is listed in the Red Data Book of Ukraine as a relic species on the limits of its range (<https://redbook-ua.org/>), and has a fairly wide distribution range in the southern, mountainous part of Crimea, growing there in populations of various sizes, often isolated from each other (Didukh 1992; Yena et al. 2005; Drescher et al. 2007). It forms monodominant communities in many areas and a significant number of the Crimean flora and fauna species are associated only or mainly with light forests of *J. excelsa* (Didukh et al. 2016; personal data of O.V. Kukushkin). Thus, the study of this representative of the Crimean flora is important for understanding the genesis of current Crimean biota characterized by striking Mediterranean features.

Natural regeneration of *J. excelsa* in some locations was not common (Korshikov and Nikolaeva 2013; Kor-

shikov et al. 2015), but the species propagated efficiently in larger populations (Drescher et al. 2007). Surveys conducted in natural reserves of Crimea showed that its genetic, morphometric, and ontogenetic diversity was maintained (Korshikov et al. 2015). Compared to the populations outside of Crimea, low levels of both morphological (Mazur et al. 2004; Douaihy et al. 2012) and genetic (Douaihy et al. 2011; Korshikov and Nikolaeva 2013) differentiation of the juniper were observed in the coastal Crimean stands, while inland localities of the species remained mostly unstudied. The origin of *J. excelsa* in Crimea and the time of isolation of this part of its range also remain an unresolved issue, and many scenarios are possible here, from the separateness of the stand from the Tertiary to migration to Crimea only at the end of the Pleistocene. The history of the species in this place, as well as its possible migrations and changes in range, are also unknown.

Using the morphometric methods, chloroplast DNA (cpDNA), and nuclear internal transcribed spacer region ITS1-5,8S-ITS2 (ITS) sequencing, we studied the variability and differentiation of *J. excelsa* within its whole distribution range in the peninsula, including coastal and inland populations. The objective of this work was: 1) to analyze phylogenetic relatedness between the Crimean junipers and individuals representing other parts of the species range, 2) to check whether the structure of morphological differentiation is congruent with the results of the genetic analyses, 3) to investigate whether ecological or geographical factors related to the location of the population can explain some variability of the species, and 4) to reveal potential demographic expansion of *J. excelsa* in the Crimean Peninsula.

MATERIAL AND METHODS

Material

Seven Crimean populations of *J. excelsa* were explored during the summer 2017, and additional material from the small, isolated and unstudied population in Chatyr

Dag was obtained in 2019. In all populations, material from 59 trees was collected (Tab. 1, Fig. 1). Altogether, 245 cones and the same number of seeds, as well as 297 small parts of shoots with leaves were examined for their morphology. The leaves of 48 individuals secured in silica-gel were used for genetic tests, because the quality of remaining samples was low (Tab. 1).

Biometry

The collected material was dried without pressing before measurement. The choice of traits analyzed (Tab. 2) was based on previous examinations by different authors, performed for *J. excelsa* and other species of juniper (Marcysiak et al. 2007; Mazur et al. 2010, 2018; Douaihy et al. 2012). Seven traits were exam-

Table 1. Sampled populations of *J. excelsa* in Crimea

Population	Geographical position	No. of specimens analyzed		Longitude N	Latitude E	Altitude	Collector
		biometry ¹	genetics				
Kara Dag	eastern	1/10 (4/4/56)	5	44°54'52.97"	35°12'55.18"	234	O. Kukushkin, Yu. Krasylenko
Sudak	coastal	12 (36)	5	44°50'32.92"	34°56'37.11"	139	Yu. Krasylenko, O. Kukushkin
Uzundza	montane	10 (50)	5	44°27'47.9"	33°52'09.4"	768–770	Yu. Krasylenko, O. Kukushkin
Fiolent	southern	5 (25)	5	44°30'15.3"	33°30'29.0"	55	Yu. Krasylenko, O. Kukushkin
Nikita	coastal	10 (50)	10	44°30'25.09"	34°14'52.28"	50	S. Sadogurska, Yu. Krasylenko
Bogatoye Ushelye	montane	4 (4)	4	44°32'05.56"	33°51'23.24"	700–770	O. Kukushkin
Laspy	southern coastal	9 (36)	10	44°25'09.25"	33°40'36.57"	50	S. Sadogurska, Yu. Krasylenko
Chatyr Dag	montane	4 (40)	4	44°48'02.63"	34°19'16.36"	650–1000	O. Kukushkin, Yu. Krasylenko

¹ in parentheses – number of measured cones, seeds, and shoots (always the same number of cones, seeds and shoots were measured with the exception of Kara Dag: 4 cones and 4 seeds from one individual and 56 shoots from 10 individuals).

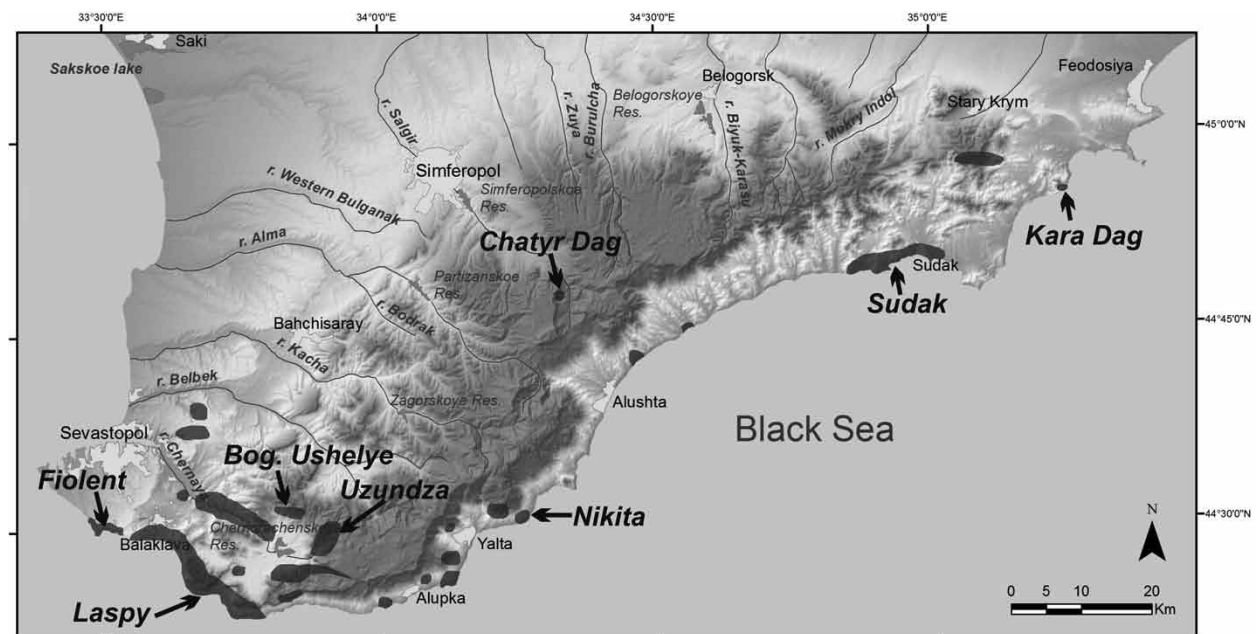


Figure 1. Geographical positions and names of sampled populations of *J. excelsa* s. str. in Crimea (arrows) against the ranges of communities where the species dominate [in grey; based on Didukh (1992) with O. Kukushkin's corrections and additions].

Table 2. Morphological characteristics used for the evaluation of the Crimean *J. excelsa* populations: mean values (M) with standard deviation (SD), minima (Min) and maxima (Max), coefficient of variation (CV), and results of comparisons between populations performed data: Kruskal-Wallis (KW) test between all populations and U Mann-Whitney (UMW) test between montane (M) and coastal (C) population and between eastern (E) and southernmost (S) populations, and basing on the set of individuals means: ANOVA and Student’s *t* test, respectively based on all data: Kruskal-Wallis (KW) test between all populations and U Mann-Whitney (UMW) test between montane (M) and coastal (C) population and between eastern (E) and southernmost (S) populations, and basing on the set of individuals means: ANOVA and Student’s *t* test, respectively

Code	Character	Mean ± SD	Min	Max	CV [%]	F ANOVA	KW P	comparison M-C		comparison E-S	
								F test t	UMW P	F test t	UMW P
CL	Length of cone [mm]	9.1 ± 1.08	6.45	12.71	11.86	3.57*	0.00*	2.32	0.29	1.03	0.25
CD	Diameter of cone [mm]	8.83 ± 1.15	6.31	13.23	13.04	3.60*	0.00*	2.11	0.46	1.21	0.09
CSN	Number of cone scales	7.59 ± 0.99	6.00	10.00	13.04	2.03	0.00*	2.15	0.68	14.99*	0.00*
SN	Number of seeds	6.04 ± 1.66	2.00	11.00	27.46	1.12	0.10	1.23	0.02*	1.30	0.74
SL	Length of seed [mm]	4.77 ± 0.59	3.19	6.37	12.32	1.86	0.00*	1.04	0.08	1.34	0.19
SW	Width of seed [mm]	3.06 ± 0.50	1.81	4.54	16.38	3.64*	0.00*	2.13	0.03*	1.55	0.00*
LN	Number of leaves	20.33 ± 4.02	12.00	34.00	19.78	5.79*	0.00*	2.57*	0.00*	1.62	0.00*
ST	Thickness of the last ramification shoot with leaves [mm]	0.76 ± 0.10	0.54	1.07	12.94	6.28*	0.00*	1.88	0.44	1.01	0.23
CL/CD	Ratio of length of cone/diameter of cone	1.04 ± 0.07	0.87	1.27	6.90	1.69	0.00*	1.00	0.00*	1.18	0.28
SL/SW	Ratio of length of seed/width of seed	1.59 ± 0.26	1.09	2.57	16.19	2.67*	0.00*	1.85*	0.00*	2.74	0.00*
CD/SN	Ratio of diameter of cone/number of seeds	1.56 ± 0.43	0.84	3.29	27.53	0.96	0.01*	1.14	0.00*	1.12	0.16
CD/SW	Ratio of diameter of cone/width of seed	2.93 ± 0.44	1.94	4.35	15.12	0.80	0.07	1.03	0.03*	1.02	0.10
CL/SL	Ratio of length of cone/length of seed	1.93 ± 0.23	1.51	2.71	11.95	0.54	0.04	1.57	0.18	2.17	0.03*
CSN/CD	Ratio of number of cone scales/diameter of cone	0.87 ± 0.13	0.58	1.27	14.63	0.76	0.01*	1.55	0.62	1.40	0.26
CD/ST	Ratio of diameter of cone/number of seeds	11.87 ± 6.85	6.85	18.77	20.48	7.54*	0.00*	3.55	0.36	2.89	0.02*
LN/ST	Ratio of number of leaves/thickness of the last ramification shoot with leaves	27.27 ± 3.64	13.64	53.97	24.29	4.11*	0.00*	1.01*	0.00*	1.35*	0.00*

* statistically significant with $p < 0.01$.

ined under the Delta Optical SZ-630 microscope: cone length (CL), seed length (SL), seed width (SW), thickness of the last ramification shoot with leaves (ST), and two measurements of the width of the cone at its widest point, perpendicular to each other, the average of which gave the diameter of the cone (CD). Leaves were counted (LN) under the microscope on the last

5 mm long section of the shoot. The Delta Optical DLT-CamViewer software was used to obtain digital images and to measure elements. The results were exported to html files, which in turn were copied to the database in Excel. Numbers of cone scales (CSN) and seeds (SN) were counted manually. Eight ratios were calculated with the measured/counted characteristics (Tab. 2).

Statistical treatment of morphological data

The symmetry and unimodality of the distribution frequency of measured characteristics were checked using Shapiro-Wilk's test, and mutual correlations of characteristics were examined using Spearman's rank correlation coefficient (Zar 1999). The basic descriptive statistics (arithmetic means, minima and maxima, standard deviations, and coefficients of variations – CV) were obtained for all data and for each population. The set of individual means was created, as a basis for further analyzes, and the normality of traits distribution was checked again. ANOVA helped to define differences between populations, after verifying the homoscedasticity of variances with the Brown-Forsythe test. Differences between groups and populations were tested by Student t test or Tukey's test, when characters fitted parametric tests, and U Mann-Whitney test or Kruskal-Wallis test, when they failed parametric tests; significance (P) was reported (Zar 1999). The influence of ecological and geographic factors on the differences between populations was investigated by comparing coastal and mountain populations as well as eastern and southernmost populations (Tab. 1). Discriminant analysis conducted for six populations (Chatyr Dag, Fiolent, Laspy, Nikita, Sudak, Uzundza) helped to verify the grouping of the populations. The Kara Dag and Bogatoye Ushelye populations, comprising only few cones, were excluded from these analyses. Traits used in this analysis had normal distributions and were chosen based on their significance in differentiating populations, showed by ANOVA, and the lack of strong correlations (Sokal and Rohlf, 2003). The software package STATISTICA 13.3 (StatSoft) was used for calculations.

DNA extraction and sequencing

An AX Plant Kit (A&A Biotechnology) was used for DNA isolation, according to the manufacturer's instruction. To screen possible genetic variation, four non-coding cpDNA fragments [*trnL-trnF* (Taberlet et al. 1991), *trnD-trnT* (Grivet et al. 2001), *trnS-trnG* (Adams and Kauffmann 2010), and *trnH-psbA* (Shaw et al. 2005)] and the nuclear internal transcribed spacer region ITS1-5,8S-ITS2 (ITS) were sequenced. Amplifications of the chloroplast regions were conducted in 9 µl reaction mixture containing 4.0 µl of Multiplex PCR Master Mix (Qiagen), 2.2 µl of RNase-free water (Qiagen), 0.8 µl of primer mixture (0.2 µM of each

primer), and 2.0 µl of diluted DNA. The total volume of the mixture for amplification of the ITS fragments was 15 µl [6.75 µl of Multiplex PCR Master Mix, 3.95 µl of RNase-free water, 1.3 µl of primer mixture (0.2 µM of each primer), and 3.0 µl of diluted DNA]. The PCR program for cpDNA fragments was according to Shaw et al. (2005); in turn, the PCR profile for the ITS amplification was described by Zalewska-Gałosz et al. (2010). After PCRs, all products were purified with the ExoBAP kit (EURx), and cpDNA products were sequenced directly using a BrilliantDye Terminator v. 3.1 Cycle Sequencing Kit (Nimagen). The ITS fragments were first run on 1.5% agarose gels in 1 × TBE buffer (4.5 h, 60 A) and stained with SimplySafe (EURx). Then, the ITS bands were excised and purified using a Gel-Out kit (A&A Biotechnology). Before visualization on an ABI 3500 sequencer (Applied Biosystems), an ExTerminator Kit (A&A Biotechnology) was used to remove unincorporated dNTPs and primers from the sequenced products. Variable sequences for different loci were deposited with the accessions numbers SAMN19591084 – SAMN19591104 within the BioProject PRJNA735631 in GenBank (Supplementary Tab. 1).

Genetic data analyses

Sequences were assembled using BioEdit v. 7.0.4 (Hall 1999) and adjusted manually, based on the GenBank accessions LC420837, LC420838, LC420839, and LC420850 of *J. excelsa* for the alignment of the *trnD-trnT*, *trnL-trnF*, *trnS-trnG*, and ITS regions, respectively. The *trnH-psbA* fragments were aligned based on the accession MH566939 of *Juniperus chinensis*.

The DnaSP program, version 6.0 (Rozas et al. 2017), was used to indicate parsimony-informative sites (P_i) in each variable cpDNA fragment and calculate the haplotype diversity (H_d) of cpDNA markers in each population. To check whether the observed frequency distributions of the mutations in the haplotypes resulted from range expansion, Tajima's D (Tajima 1989) as well as Fu's F_s test (Fu 1997) and Fu and Li's D^* and F^* statistics (Fu and Li 1993) were calculated using DnaSP. Range expansion is likely when Tajima's $D < 0$, F_s is significantly negative, while D^* and F^* parameters are not significant (Fu 1997). Additionally, mismatch distribution analysis (distribution of the observed number of differences between pairs of haplotypes) was carried out in DnaSP to test potential current expansion

in the *J. excelsa* populations. Arlequin, version 3.5.2.2 (Excoffier and Lischer, 2010), was used to construct the minimum spanning tree for cpDNA haplotypes. In the construction of this network, only parsimony-informative sites were used.

Phylogenetic analyses were conducted using a maximum likelihood (ML) method implemented in MEGA X version 10.1 (Kumar et al. 2018) and Bayesian inference (BI) in MrBayes 3.2.6 (Ronquist et al. 2012), based on the two data sets. The first set contained concatenated *trnD-trnT/trnL-trnF/trnS-trnG* fragments derived from 10 *J. excelsa* individuals representing different cpDNA haplotypes in the present study as well as seven accessions from GenBank with herbarium numbers: 14742(BAYLU), 13721(BAYLU), 14155(BAYLU), 8785(BAYLU), 9433(BAYLU), 13720(BAYLU) and 8786(BAYLU) (Supplementary Tab. 2), another set included the ITS sequences. The *trnD-trnT*, *trnL-trnF*, and *trnS-trnG* fragments were chosen because they were also amplified and showed polymorphism in some other *J. excelsa* specimens inhabiting different parts of the species range (Hojjati et al. 2018) and one *J. chinensis* (Mao et al. 2010). For the ITS tree constructions, nine sequences from this study as well as 12 *J. excelsa* (LC420820, LC420840, LC420855, LC420800, LC420850, LC420830, LC420810, LC421168, LC421145, LC421218, LC421203, LC421035), two *J. communis* (LC420860, LC420950), and two *J. chinensis* (LMH566939, FJ980279) sequences from GenBank were used (Supplementary Tab. 1, 2). The best-fit models of nucleotide substitution for ML analyses were based on the Akaike information criterion (AIC) and tested with MEGA. Tamura 3-parameter (T92; Tamura 1992) and Hasegawa-Kishino-Yano (HKY; Hasegawa et al. 1985) models were indicated for cpDNA and ITS fragments, respectively. The reliabilities of ML trees were inferred using bootstrapping with 1,000 replicates. To construct BI trees, the GTR substitution model with gamma-distributed rate variation across sites and a proportion of invariable sites was implemented. Two independent runs of 10 million generations each were carried out, with each run consisting of four chains (three heated, one cold). Trees were sampled every 1,000 gen-

erations. A majority-rule consensus tree with posterior probabilities (PPs) from two runs was produced after excluding the first 25% of runs as burn-in.

RESULTS

Morphological characteristics

In the entire data set, unimodal frequency distributions of values were found for CL, CD, SL, SW, ST, CL/CD, CSN/CD, and slightly skewed for SL/SW, CD/SW, LN/ST, and CD/ST. The characteristics CSN, SN, LN, CD/SN, CL/SL were not normally distributed. In the set of all individual means, the frequency distributions were unimodal for all traits, and their variances were homoscedastic. The variation of the studied characteristics was moderate. Of all parameters, CD/SN showed the highest variation (Tab. 2), with CV > 25% in every population. The trait CL/CD was the most stable one, with CV values ranging from 2.61% in Nikita to 7.75 in Laspy (Supplementary Tab. 3). The examined characteristics were moderately correlated. The strongest correlations ($r > 0.8$) were found for CL and CD, SN and CD/SN, and LN and LN/ST. Strong relations, with $r > 0.6$, occurred between CL and CD/ST, ST and CD/ST, SW and SL/SW, and SL/SW and CD/SW. Leaf and shoot characteristics (LN and ST) were not correlated with features of cones and seeds (Supplementary Tab. 4).

Table 3. Number of individuals (N_{ind}), haplotypes of two polymorphic cpDNA sequences: *trnD-trnT* and *trnH-psbA* and haplotype diversity (H_d) in the populations of *J. excelsa* sampled in the Crimean Peninsula

Population	N_{ind}	Number of cpDNA haplotypes										H_d
		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	
Kara Dag	5	4	–	1	–	–	–	–	–	–	–	0.400
Sudak	5	3	–	–	–	–	–	–	–	1	1	0.700
Uzundza	5	3	–	–	–	–	–	1	1	–	–	0.700
Fiolent	5	2	1	–	–	1	1	–	–	–	–	0.900
Nikita	10	8	2	–	–	–	–	–	–	–	–	0.356
Bogatoye Ushelye	4	4	–	–	–	–	–	–	–	–	–	0
Laspy	10	7	1	1	1	–	–	–	–	–	–	0.533
Chatyr Dag	4	4	–	–	–	–	–	–	–	–	–	0
Total	48	35	4	2	1	1	1	1	1	1	1	

Differentiation among populations based on morphology

The results of ANOVA performed on the set of individuals means revealed that CL, CD, SW, LN, ST, SL/SW, CD/ST, and LN/ST features with $p < 0.05$ were responsible for differentiating populations; however, according to Tukey's test results, some of them differed only between single populations. Uzundza, Sudak, and Chatyr Dag differed most from other populations (Supplementary Tab. 5). Based on the results of Student's t test performed for individuals means, the coastal and mountain groups of populations differed significantly in terms of: LN, SL/SW, and LN/ST. This test, when performed for eastern and southernmost populations, showed significant differences in CSN and LN/ST. Non-parametric tests, applied to all data, showed a greater number of significant differences (Tab. 2).

The discriminant analysis based on the traits: CL, CD, SW, and ST, showed grouping of populations: Fiolent, Laspy, and Nikita in the center of the graph, while the remaining three populations took the position outside this group (Fig. 2a). On the plot of all cases, one rather loose cloud was visible, but the outside located populations were more compact, and ellipses of the

confidence area (95%) of Chatyr Dag and Uzundza only slightly overlapped each other (Fig. 2b). The first discriminant variable was most strongly connected with ST and CD, and therefore, the separateness of Chatyr Dag depended on these features. The second variable was determined mainly by SW, and the differentiation of Sudak from Uzundza was based on these traits.

Diversity of molecular markers, phylogenetic analyses and demographic history

Four cpDNA sequences were obtained for each *J. excelsa* individual, and the total length of the combined *trnL-trnF/trnH-psbA/trnD-trnT/trnS-trnG* sequence was 2,643 nucleotides. The *trnL-trnF* and *trnS-trnG* markers showed no polymorphisms; thus, 10 cpDNA haplotypes were established based on the variation of *trnD-trnT* and *trnH-psbA* fragments. In these fragments, 2 and 10 variable sites were found, respectively. Two sites in the *trnD-trnT* and five sites in *trnH-psbA* regions were parsimony-informative (P_i). The most frequent one was the H1 haplotype (72.9%), which was found in all populations (Tab. 3). The H2 haplotype (8.3%) occurred in three populations, and the H3 haplotype (4.2%) was noticed in two localities. The

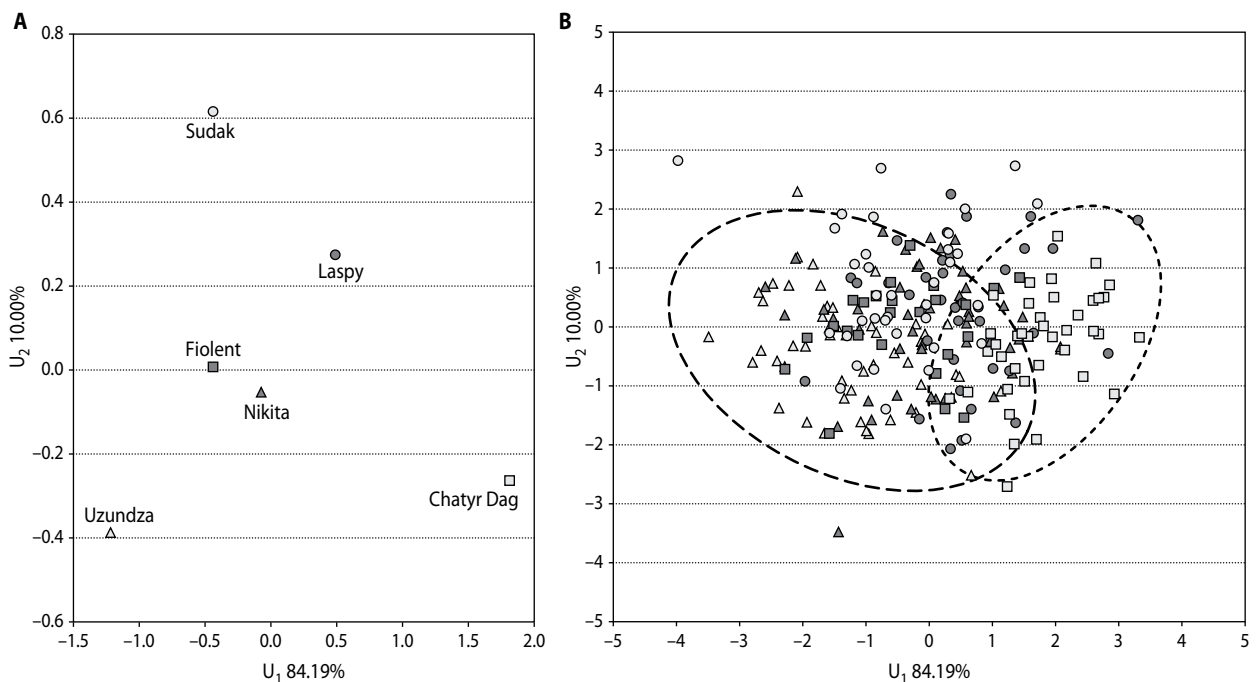


Figure 2. Results of discriminant analysis of six populations of *J. excelsa* from Crimea based on morphological traits. A – Scatterplot showing populations means, B – Scatter of all cases.

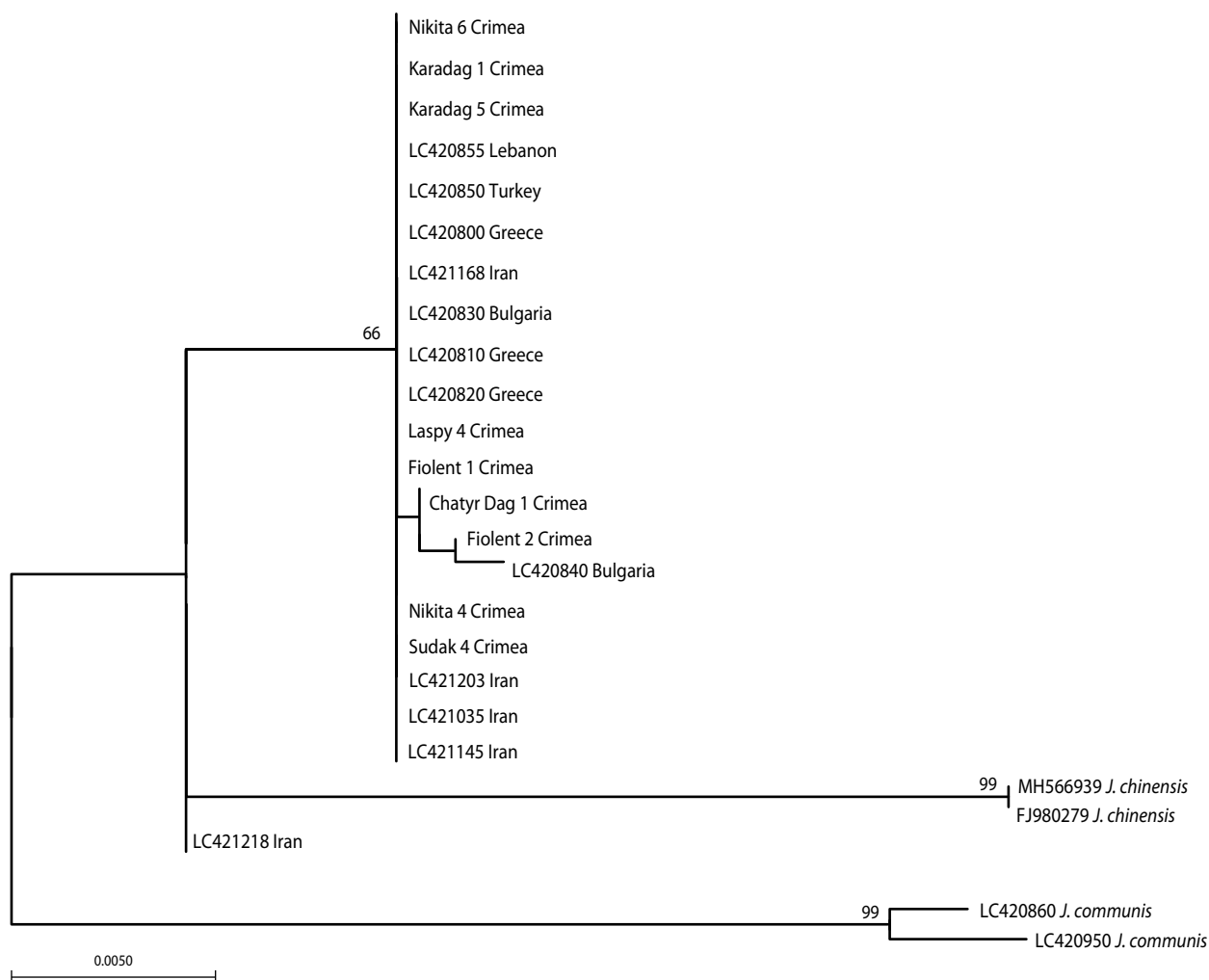


Figure 3. Maximum likelihood (ML) tree based on the nuclear ITS sequence of the *J. excelsa* individuals. Samples from the Crimean Peninsula are listed in Supplementary Tab. 1. GenBank accessions of other *J. excelsa*, *J. chinensis* and *J. communis* are included (Supplementary Tab. 2). The bootstrap values $\geq 50\%$ are shown only (1,000 replicates)

remaining seven haplotypes were observed only once. Haplotype diversity ranged from $H_d = 0$ in both Bogatoye Ushelye and Chatyr Dag to 0.9 in Fiolent. In the minimum spanning tree, the central position was occupied by the H1 haplotype (Supplementary Fig. 1). The remaining haplotypes differed from H1 maximally by three mutational steps.

Among the tests conducted to detect potential range expansion, only Fu's F_s value was significantly negative ($F_s = -2.813$, $P = 0.036$), and Tajima's D (-1.596 ; $0.1 > P > 0.05$), Fu and Li's D^* (1.424 ; $0.1 > P > 0.05$), and F^* (0.430 ; $P > 0.1$) were nonsignificant. Mismatch analysis displayed a unimodal distribution (Supplemen-

tary Fig. 2), which can imply a recent demographic expansion.

The ML and BI phylogenetic trees were almost identical to one another for both ITS cpDNA sequence sets; thus, only the ML trees are shown and discussed. Phylogenetic analyses were based on 2,113 nucleotide sites of the concatenated *trnD-trnT*, *trnL-trnF*, and *trnS-trnG* cpDNA fragments. In general, analysis of the cpDNA regions showed that the *J. excelsa* haplotypes described in the Crimean Peninsula formed one group with *J. excelsa* from Greece, Bulgaria, Turkey, and Lebanon (Supplementary Fig. 3). In this group, only samples representing H2, H5, and H6 haplotypes, found in

the Fiolent, Nikita, and Laspy localities, were distinct to some extent and formed two subgroups within the main cluster with 61 and 62% bootstrap supports; however, one subgroup included a sample from Greece.

It was difficult to obtain nuclear ITS sequences of good quality, and only nine fragments representing *J. excelsa* individuals from Kara Dag, Sudak, Fiolent, Nikita, Laspy, and Chatyr Dag populations were used (Supplementary Tab. 1). The total length of the ITS alignment was 616 nucleotides. In the ITS tree, all *J. excelsa* samples from Crimea clustered with the majority of individuals of the same species from other geographical locations (66% bootstrap support; Fig. 3). Two accessions of *J. chinensis* (MH566939 and FJ980279) and two *J. communis* (LC420860 and LC420950) were significantly distinct (99% bootstrap supports) from the *J. excelsa* ingroup.

DISCUSSION

Phylogenetic relatedness between *J. excelsa* s. str. from Crimea and other parts of the species range

Phylogenetic analyses of the cpDNA fragments revealed that the *J. excelsa* samples from the Crimean Peninsula were closely related to the individuals from other parts of the species range: Bulgaria, Greece, Turkey, and Lebanon. In general, this result was supported by the ITS phylogenetic investigation as the majority of *J. excelsa* individuals fell into one group, although the bootstrap value was not high. This observation confirms the statement of Adams et al. (2016) that *J. excelsa* is a genetically uniform species. A significant genetic similarity of the *J. excelsa* populations occupying different areas can result from a shared ancestry and indicate that the species range was more continuous in the past, with only recent fragmentation (Yena et al. 2005; Cordova 2007). According to Adams et al. (2014, 2016), only *J. excelsa* samples from Lebanon were different to some extent, and they formed a strongly supported subgroup in the phylogenetic tree based on the nuclear ITS and cpDNA markers. The molecular differences of the Lebanese specimens were supposed to be an effect of gene inflow from *J. polycarpus* into *J. excelsa*, as these two species co-occur in this region (Adams et al. 2016). However, one [14155 (BAYLU)] of the Lebanese accessions used by Adams et al. (2014, 2016) clustered

with other *J. excelsa* samples in the analyses of Hojjati et al. (2018) as well as in the present study. In turn, Hojjati et al. (2018), who investigated genetic relationships between the *Juniperus* populations in Iran using nuclear and cpDNA loci, found that some Iranian samples classified as *J. excelsa* were related to *J. polycarpus* var. *turcomanica*, others to *J. seravschanica*. The authors concluded that there was no *J. excelsa* s. str. in Iran, which has previously been suggested by Adams et al. (2014). In our ITS tree, four Iranian accessions (LC421168, LC421203, LC421035, and LC421145), were placed with other *J. excelsa* samples, while the LC421218 individual was clearly different. Most probably, this unexpected similarity of some ITS sequences from Iran to other accessions of *J. excelsa* in the present work can result from the relatively short ITS fragments studied and the small number of variable sites.

Morphological differentiation of *J. excelsa* s. str. in Crimea versus genetic diversity and environmental influences

Multivariate analyses pointed out similarities between Fiolent, Nikita, and Laspy, that is, populations located in the southern part of the Crimean coast, at about 50 m above sea level. These three populations took the central positions on the scatterplots, and their variability was quite high as their confidence ellipse covered a large part of the whole variability. Additionally, the H2 cpDNA haplotype, being the second most common haplotype, was only found in the Fiolent, Nikita, and Laspy localities. It is likely that the substantial morphological variation and the presence of H2 haplotype in three southernmost populations result from their common ancestry, indicating that they can be remnants of a huge continuous population in the past.

Although the analysis showed little morphological difference between the groups of mountain and coastal populations, two of the mountain populations, Chatyr Dag and Uzundza, differed by the greatest number of features from all the others. The Chatyr Dag population, with the smallest and elongated cones and seeds, grows in the most severe climate conditions among all Crimean localities of the Greek juniper, and is isolated from others. Therefore, its distinctiveness may be caused by climatic conditions, as the effects of climate on the growth and diversity of junipers have already been described (De Soto et al. 2014; Pellizzari et al. 2014; Ma-

zur et al. 2016). The other possibility is the loss of variability as a result of the decline of this population, what can be enhanced by the lack of cpDNA haplotype diversity in Chatyr Dag. The Uzundza population grows in mountains, but in the milder climate, and the distances between this population and its neighbors are smaller, when compared to the Chatyr Dag population. Despite this, morphological differences between Uzundza and the closest populations in Laspy and Nikita were found. Its haplotype diversity is quite substantial, which results from the presence of unique haplotypes, H7 and H8. Again, the morphological distinctiveness of this population can be explained either by the effect of the climate, that seems to be here most favorable to the species, or by genetic drift acting on this isolated locality. The comparison between the populations from the eastern and southern ends of the range of *J. excelsa* in the Crimea revealed few differences, but Sudak being the largest among the eastern populations differed in several features from all the others. The population was also genetically distinguished by having two unique haplotypes, H9 and H10.

Origin and dynamics of *J. excelsa* in Crimea

The minimum spanning network constructed based on the parsimony-informative sites found in the *trnH-psbA* and *trnD-trnT* cpDNA regions in the *J. excelsa* individuals from the Crimean Peninsula represented a typical starburst topology, suggested for an abundant species that expanded recently from a small number of individuals (Avice 2000). In such networks, the central position is occupied by an ancestral haplotype, which is the most common and widespread haplotype in the studied area; the remaining haplotypes are assumed to be recent derivatives connected to the central one mostly by short branches (Avice 2000). In the Crimean part of the *J. excelsa* range, the H1 haplotype was found in each locality and its contribution was the highest; thus, it seems to be ancestral. Except for H2 and H3 haplotypes, all others occurred once in different populations. Previous minimum spanning networks including the *J. excelsa* accessions involved also additional *Juniperus* species and varieties from Europe and Asia. However, the *J. excelsa* haplotypes differed from one another by one to three mutations (Adams et al. 2014, 2016). On the other hand, the results of analyses performed to test signatures of demographic expansion of *J. excelsa* in the

Crimean Peninsula were contradictory. The distribution of the observed number of differences between pairs of haplotypes was unimodal, implying recent expansion. Significantly negative value of Fu's F_s and non-significant values of Fu and Li's D^* and F^* also seemed to confirm a recent expansion scenario. In turn, Tajima's D value was non-significant, albeit negative, and this did not agree with the expansion model. We think that lack of concordance between different tests conducted to detect the demographic history of Crimean populations of *J. excelsa* can result from the low level of polymorphism in the cpDNA fragments studied. Similar outcomes and conclusions have been described for the two vitex species *Vitex rotundifolia* and *V. trifolia* in East Asia, both representing distributions under an exponential growth rate model, but Tajima's D and Fu's F_s tests results were non-significant (Sun et al. 2019).

The homogeneous character of *J. excelsa* s. str. within the whole range, confirmed previously by several authors and in our study, indicates the same origin of its many scattered populations. The traces of the Mediterranean flora in Crimea could be dated back to the Miocene-Pliocene (Didukh 1992; Cordova 2007), and it is assumed that the Crimean Peninsula could have served as a refuge for thermophilous species during the cold stages of the Pleistocene (Didukh 1992; Cameron et al. 2013). During the Pleistocene, in Crimea, the ranges of species were changing not only due to temperature fluctuations, but also because of changes in the level of the Black Sea, which remained low until the Holocene, what enabled plants migrations (Cordova 2007; Krijgsman et al. 2019). The following sea level rise is estimated for about 7,500 years BP (Cordova 2007; Fouache et al. 2012), which is consistent with the beginning of the Atlantic period. It is supposed that in this time, *J. excelsa* was abundant on the Crimean coastal zone (Yena et al. 2005), which could explain the ancestral character of its southern coastal populations in Crimea, proposed in our study based on the morphological similarities of these populations and the presence of the cpDNA H2 haplotype. A significant part of the Crimean coast with Mediterranean flora was eventually flooded, and the possibilities for plant migration around the Black Sea decreased (Yena et al. 2005). This scenario can be confirmed by the results of our phylogenetic analyses, which implied the common origination and recent fragmentation of populations of the species. These warm

conditions could also foster the expansion of *J. excelsa* in Crimea northward and to higher locations in the mountains, which is consistent with the recent expansion model revealed by the minimum spanning network.

CONCLUSIONS

Our molecular analyses of nuclear ITS and four cpDNA markers revealed low level of genetic diversity in eight Crimean localities of *J. excelsa*, and close relationship of these stands with individuals inhabiting other parts of the species range. Morphological investigations also showed only minor differences between populations located in different parts of the peninsula. These results are congruent with the statement of Adams et al. (2016), who postulated that *J. excelsa* was a uniform species. Relatively substantial morphological variation and the share of cpDNA H2 haplotype in Fiolent, Laspy, and Nikita can suggest that these three southernmost populations arose by recent fragmentation of the juniper's continuous range. In turn, genetic drift and/or selection pressure acting through local climatic conditions can be responsible for differences between Chatyr Dag, Uzundza and Sudak populations.

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