Sequencing conserved region of endangered species *Celtis caucasica* Willd

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Abstract. The rarest species of relict ironwood *C. caucasica* Willd. is naturally occurred on the Ile-Alatau Mountains but very little is known about genetic diversity and distribution and size of its populations in Kazakhstan. In during the sampling expedition were found two additional plant populations in Dzungarian and Kyrgyz Alatau Ranges. The objective of this work was targeted towards sequencing ITS region of *C. caucasica* and compare the obtained nucleotide sequence with available data on NCBI GeneBank for confident species identification. The identity of *C. caucasica* sequence and available *C. australis* and *C. bungeana* sequences was 93.87% and less. It could be associated with absence sequences producing significant alignments with the studied ironwood sequence and important deposited sequences of GenBank lacking Latin binominals is from environmental samples. Clarifying taxonomical status species and subspecies is difficult by morphological data and molecular markers should be used to correct identifying an endangered species of *C. caucasica* growing in the east-southern and the south regions of Kazakhstan and providing direction to the conservation management of the plant.

1 Introduction

Kazakhstan is the ninth largest country in the world in terms of its area, but more than 75% of its territory belongs to a dry arid zone with a sparsely wooded and treeless landscape. In general, there is 4.2-4.6% of the territories of the Republic covered by the forest [1, 2]. So, the creation of tree plantations on the territory of forest-deficient regions is of great ecological importance.

Kazakhstan's flora contains more than 5754 species of vascular plants [2, 3]. Among them, 68 arboreal, 226 shrub and 433 semi-shrub plant species are naturally occurred. Studying a natural genetic diversity of dendroflora has great practical issue and allow to increase and to improve species content of green plantings, and find resistant genotypes and new deciduous ornamental trees for city environments [4].

One of recommended species for particulate capturing in the big megapolis could be nettle tree (*Celtis australis*) because of its rough leaf surface [5]. Although, certain species featuring small leaf surface (needles), for instance the Scots pine *Pinus sylvestris* L. is the most common plants used for larger forestation. The three species of conifers *P. sylvestris*

L., *Juniperus communis* L. and *Picea abies* L. (Karst.) are used mainly in the landscaping of Moscow [6]. In Moscow, 366 species and forms of woody plants used in

different types of urban plantings have been identified [4]. Whereas the assortment of woody plants in Rostov-on-Don, belonging to the steppe zone of Russia, consists of 211 species, one of which is the widespread *C. occidentalis* L. (Northern Hackberry, American Hackberry, Common Hackberry). It is considered that the American Hackberry is an introduced species in Ukraine, the North Caucasus and Russia [7]. An analysis of the collection in the Donetsk Botanical Garden (DBS) showed a tendency to run wild and spontaneous distribution of more than 40 tree species, including *C. occidentalis* L. [8].

The Hackberry is also found in the walnut forests of the Western Tien Shan belonging to the territory of the Central Asian states [9, 10]. In Kazakhstan, the rarest species of relict ironwood (Caucasian Hackberry) *C. caucasica* Willd. (1806) was collected by geobotanist, academician B.A. Bykov in the Ile-Alatau State National Natural Park (SNNP). It was accepted that *C. caucasica* has the northern border of distribution in the Dzungarian Alatau [11, 12].

The objective of this work was targeted towards sequencing ITS region of *C. caucasica* and compare the obtained nucleotide sequence with available data on NCBI GeneBank for confident species identification.

2 Materials and methods

2.1 The collection of samples and DNA isolation

Plant samples of *C. caucasica* collected from populations locating in the mountain ravine of Major Almatynka near Almaty city. Genomic plant DNA isolation is provided from young fresh collected leaves samples in 3-5 repeats using the commercially available kit DNeasy Plant MiniKit (Qiagen, #69104). The quality of DNA samples were visual evaluated by using an electrophoresis technique and 0.9% agarose-gel containing ethidium bromide.

2.2 DNA amplification

The ITS region was amplified on the thermocycler Mastercycler pro (Eppendorf) with using universal specific primer pair ITS1-F 5'- TCCGTAGGTGAACCTGCGG and ITS4-R 5'- TCCTCCGCTTATTGATATGC (White et al., 1990). The PCRs were performed in volume of 25 ul and contain 2.5µl of 10 × dNTPs (2 mmol/l each nucleotide), 2.5µl of 10× PCR buffer, 2.5µl of 25mM Mg2+, 1U of Taq DNA polymerase (ThermoScientific), 0.5 µl of 10 pmol/µl each primer and 5 µl of DNA target (0,02 µg/µl). PCR programme was 95^0C for 5 min, followed by 35 cycles of 30 sec at 94^0C, 40 sec at 58^0C and 50 sec at 72^0C, with a final elongation step of 7 min at 72^0C. The PCR products were examined via electrophoresis in 1.2% TAE-agarose gel and ChemiDoc XRS+ (BioRad, USA).

2.3 Sequencing and analysis

According to manufacturer's protocol the PCR products were purified by using Exonuclease I (Fermentas) и phosphatase (Fastap, Fermentas). Sequencing reactions were done with BigDye terminator v3.1 sequencing kit and DNA fragments were fractionated on 3730x1 DNA analyzer (Applied Biosystems, USA). Obtained nucleotide sequences were analyzed by SeqMan (Applied Biosystems). To identify plant species, the nucleotide sequence was compared with the nucleotide sequences available through international databases GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3 Results and discussion

Apart of preliminary *C. caucasica* surveys, very little is known about genetic diversity and distribution and size of its populations. Since XIX century the territory of the Ile-Alatau State National Natural Park (SNNP) belonged to Ile-Alatau Mountains was well known as a range and habitat for the hackberry (ironwood) in Kazakhstan. Biological properties and characteristics of this population of *C. caucasica* is well studied and shown that is growing on 1400 meters of elevation where is occurred a deciduous forest, cereal grasses and shrubs [13].

Fig. 1. Geographic map of *Celtis caucasica* populations.

There is less information about other populations of *C. caucasica* that we met on the mountain passageway called Arkharly belonging to Dzungarian Alatau Range as seen on the figure (cycled area). On the figure is shown a map created via GoogleMyMap (https://www.google.com/maps/) and there is an area of Kyrgyz Alatau having opposite direction from Almaty city to Dzungarian Alatau Range. The studied populations of *C. caucasica* from Kyrgyz Alatau Range is distributed on the mountain ravine of Merki near with same named city and located more than 370 km far from Almaty city.

In all eukaryotic kingdoms, internal transcribed spacer (ITS) region is applicable for identification of the broadest possible taxonomic groups. On the table 1 showed the obtained nucleotide ITS sequence for *C. caucasica* comparing with genome sequences available in GenBank database.

Table 1. Comparing ITS sequence of *C. caucasica* with sequences of GeneBank.

Sequence 5'-3'	Accession	Description	% of identity
TGGGGTCGCGTTGGAGCG CCGAGTTAATGGCGCGAT TGGGGGTCTCCGAGGGTC TCGCGACGTGCTAAAGCG CGCGCAACGGGGCACCGA ATGCTCGACAACCACCGA ATGTTGCGGCGCTCGCAA CCGAGGACTCGTTTTTGGG CCAACCGCGAGGCTAATC	MN381774.1	Celtis australis isolate Velzen, R. van 12, ITS1, partial sequence; 5.8S *r*RNA, ITS2, partial sequence	93.87
	MN381773.1	Celtis bungeana isolate J_submit_003, ITS1, partial sequence; 5.8S *r*RNA gene, complete sequence; and ITS2, partial sequence	93.87

GCGCACGGGAGGCCAATT TCCGCCCCCGCCCGAACG CGCCCTCTCGGAGCGAGG TGCGGGGGGGGGGGACAA TGTGTGACGCCCCCGCAG ACGTGCCCCCCGCCTAATG GGTTCTGGGGCAACTTGTG TTCAAAAACTCGATGGTT	MN381776.1	Celtis choseniana isolate J_submit_005, ITS1, partial sequence; 5.8S ribosomal RNA, ITS2, partial sequence	93.59

As seen on table 1, the identity of *C. caucasica* sequence and available *C. australis* and *C. bungeana* sequences was 93.87% and less. It could be associated with absence sequences producing significant alignments with the studied ironwood sequence and important deposited sequences of GenBank lacking Latin binominals is from environmental samples.

The taxonomists have questioned with reliable identification of the plant species as well as resolving problems around *Celtis* spp. hybridizations [14]. Previously *Celtis* genus was included to the family of *Ulmaceae* Mirb. s.s.and then to separate *Celtidaceae* Link but due to phylogenetic studies the APG III system places it in the family *Cannabaceae* [15]. S.K. Czerepanov reported 5 hackberry species *C. australis* L. (Mediterranean hackberry, European nettle tree, lote tree), *C. caucasica* Willd., *C. glabrata* Stev ex Planch, *C. tournefortii* Lam. (*C. aspera* (Ledeb.) Stev.) and *C. tupalangi* Vass. [16]. However now the genus consists of about 70 species where *C. caucasica* has five synonyms *C. arcata* Buch.-Ham. ex Wall., *C. australis ssp. caucasica* (Willd.) C.C. Townsend, *C. caucasica ssp. caudata* (Planch.) Grudz. and *C. tupalangi* Vass.

Depending on taxa, ITS can had the most resolving or lower power for species discrimination than other nuclear ribosomal markers such as SSU (18S nuclear ribosomal small subunit rRNA gene) and LSU (28S nuclear ribosomal large subunit rRNA gene). Microsatellite markers have found to be ideal for plant studies. SSRs are extremely useful to assess levels of genetic similarity among closely related species, to quantify a diversity and estimate the degree of relatedness amongst different populations and between genotypes [17]. Clarifying taxonomical status species and subspecies is difficult by morphological data and molecular markers should be used to correct identifying an endangered species of *C. caucasica* growing in the east-southern and the south regions of Kazakhstan and providing direction to the conservation management.

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