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Identification and genetic diversity of *Acer ibericum* (Aceraceae) in South Caucasus

Juraj Paule[1], Nani Goginashvili[2], Irina Tvauri[2], Ladislav Paule[3], Ekaterine Gabashvili[4], Mamuka Kotetishvili[4], Nato Kobakhidze[5]*

[1]*Department of Botany and Molecular Evolution, Senckenberg Research Institute and Natural History Museum, Frankfurt/Main, Germany*

[2]*Agroforestry Research Division, Scientific-Research Center of Agriculture, Tbilisi, Georgia*

[3]*Department of Phytology, Faculty of Forestry, Technical University, Zvolen, Slovakia*

[4]*Bioinformatics Core, Scientific-Research Center of Agriculture, Tbilisi, Georgia*

[5]*Faculty of Agrarian and Natural Sciences, Agricultural University of Georgia, Tbilisi, Georgia*

**Corresponding author: jpaule@senckenberg.de*

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Abstract: Internal transcribed spacer (ITS) sequence was tested in *Acer ibericum* for its suitability as a barcoding as well as population genetic marker. The marker was reliably used to identify *A. ibericum* as well as to gain preliminary insights into the genetic diversity of Georgian populations. MegaBLAST searches of studied samples against the GenBank database revealed that all studied accessions could be considered *Acer ibericum*. Populations from Vashlovani National Park revealed the presence of unique ribotypes, and together with the population from Shavi Mountain, they were identified as the most genetically diverse ones. Based on presented data the populations from Eastern Georgia should be prioritized if conservation measures are planned.

Keywords: barcoding; genetic diversity; Georgia; internal transcribed spacer (ITS); maple

Iberian maple (*Acer ibericum* M. Bieb.) was first described by Friedrich August Marschall von Bieberstein (1808) and was named after Iberia (Kingdom of Kartli). It is distributed in the Caucasus, northeastern Turkey, and northern and northwestern Iran (Yaltirik 1967a, b; van Gelderen et al. 1994; Grimm, Denk 2014; Khademi et al. 2016). In Georgia, it is mainly distributed in the southeastern part on the massifs of Chachuna, Chatmi and Shavi mountains, in the Vashlovani basin, in the lower basin of the Alazani river as well as in southern Georgia (Bolnisi, Tetritskaro) and due to a small and fragmentary distribution range, it is considered vulnerable (VU) by the Red List of Georgia (Tarkhnishvili, Chaladze 2013).

Acer ibericum is a small tree usually up to 8 m high (but sometimes also up to 12–15 m) and it grows in a form of individual trees or small groups in a dry open woodland habitat at the altitudes of 100–1 000 m (Dolukhanov 1948; Lachashvili et al. 2007; Akhalkatsi 2010). According to de Jong's (1994) classification, *A. ibericum* belongs to the section *Acer* series *Monspessulana* (Pojárkova 1949), which roughly corresponds to the clade B of Grimm et al. (2007). In comparison with the other species of the series, *A. ibericum* displays dimorphic leaves that

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are 5-lobed in juvenile plants and sucker shoots, and 3-lobed in older plants whereas *A. monspessulanum* has distinct small, 3-lobed leaves, and its close relative *A. hyrcanum* normally has 5-lobed leaves (Grimm et al. 2007). In comparison with *A. monspessulanum* the leaf blade in *A. ibericum* is more divided and the middle lobe is broadly ovate (Yaltirik 1967b). Additionally, wax glaucosity of the abaxial leaf surface is found in all species of the series, but absent in guard cells of *A. ibericum* (Grimm et al. 2007). However, these characters might be difficult to assess in the field, especially when only e.g. one developmental stage and individual trees are present.

In 1967 Yaltirik provided a new nomenclatural combination of *A. ibericum* as subspecies of *A. monspessulanum (A. m.* subsp. *ibericum*/ M. Bieb/Yaltirik) (Yaltirik 1967a), which was later adopted by several monographs (e.g. van Gelderen et al. 1994), floras (Yaltirik 1967b) or checklists (WFO 2020). However, with the onset of molecular phylogenetics concerns about the taxonomic position of *A. ibericum* were expressed as the analyses based on the sequences of the internal transcribed spacer (ITS) of the nuclear rDNA cluster supported a separate species rank for *A. ibericum* (Grimm et al. 2006). Two length variants of ITS were found in *A. ibericum* and revealed diagnostic at the species level, positioning Iberian maple in a sister clade apart from *A. monspessulanum* and *A. hyrcanum* (Grimm et al. 2007; Grimm, Denk 2014; Khademi et al. 2016).

DNA-barcoding is widely used to identify organisms using genetic markers and within e.g. International Barcode of Life (iBOL) project it has been shown that identification is possible for a great number of taxa (Ratnasingham, Hebert 2013). For animal barcoding, the cytochrome c oxidase I (cox1 or COI) locus of the mitochondrial genome is universally used while in plants the same locus is not informative, thus alternatives are required (Fazekas et al. 2009; Hebert et al. 2003). Interestingly, ITS has been proposed for some taxonomical groups or geographical regions as a suitable marker (e.g. Chen et al. 2010). Particularly in Aceraceae, it has been demonstrated that ITS marker shows the highest interspecific and intraspecific divergences, as well as the highest species resolution of all tested single DNA barcode candidates (Han et al. 2016).

Given the DNA-barcoding suitability of the ITS for the whole family Aceraceae as well as the diagnostic properties concerning *A. ibericum* it seems like an optimal marker to differentiate *A. ibericum* from other *Acer* species. Moreover, two size variants and several single nucleotide polymorphisms (SNPs) recovered previously potentially enable to study *A. ibericum* beyond the species level as so far no population genetic characterization has been available. This would allow an assessment of e.g. genetic diversity, differentiation, and/or gene flow among populations and thus guiding the conservation strategies for this vulnerable species.

Accordingly, in this study we aimed to answer the following questions: (1) Could sampled populations morphologically identified as *A. ibericum* be identified as such based on the previously published diagnostic ITS sequence data? (2) What is the genetic diversity in studied populations of *A. ibericum*? (3) Is it possible to suggest more effective conservation strategies for *A. ibericum* in Georgia based on ITS data?

MATERIAL AND METHODS

Plant material. In total, 71 individuals representing 6 populations were investigated with 5–19 individuals per population (Table 1, Figure 1). Samples collected within a 2 km radius were considered one population. Identification of *Acer ibericum* was based on the leaf blade shape. At least one herbarium specimen was prepared from each locality. Herbarium specimens are deposited at the National Herbarium of Georgia (TBI). The coordinates for the field-collected material were obtained using a handheld GPS unit.

DNA extraction, PCR amplification, and sequencing. Total genomic DNA was extracted from silica gel-dried leaf tissue. DNA was extracted with the QiagenDNeasy® Plant Mini Kit (Hilden, Germany) from leaf fragments of approximately 1 cm^2 size, following the manufacturer's protocol. Extracted DNA was dissolved in 150 µL of elution buffer and 2 units of (U) RNase (10 $mg \cdot mL^{-1}$) were added.

The internal transcribed spacer region of the nuclear ribosomal DNA (ITS) was amplified using 2 µL of DNA template in 25 µL reactions together with 1 µL of 10 µM primers ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS7A (5'-GAG TCA TCA GCT CGC GTT GAC TA-3') using illustra PuReTaq Ready-to-Go Beads© (GE Healthcare Life Sciences, Germany). PCR reactions were performed on a Mastercycler® pro (Eppendorf,

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Table 1. Sampling localities of newly studied as well as previously published *Acer ibericum* populations, data from populations ib1-ib4 are taken from Grimm et al. (2007)

Population	Locality	*Nb*	Latitude	Longitude	Altitude (m)
Pop 1	Georgia, municipalty of Tbilisi, Betania monastery, the gorge of Samadlo	16	41.6905	44.6114	780
Pop 2	Georgia, municipality of Bolnisi, vicinity of the village Disvelis	18	41.4994	44.5508	647
Pop 3	Georgia, municipality of Tetritskaro, vicinity of the village of Samshvilde	5	41.5108	44.4870	843
Pop 4	Georgia, municipality of Dedoplistskaro, Kashebi	8	41.3119	46.5802	715
Pop 5	Georgia, municipality of Dedoplistskaro, Shavi mountain	19	41.2658	46.6356	813
Pop 6	Georgia, municipality of Dedoplistskaro, village Kasristskali	5	41.2832	46.4689	597
ib1	Georgia, N of Alpadara	4	41.2463	46.6290	
ib2	Georgia, Vashlovani	3	41.1290	46.6032	
ib3	Armenia, Norawank	2	39.6857	45.2323	
ib4	Georgia, Alpadara	2	41.1976	46.6338	

Pop – population code; *Nb* – number of studied individuals/available sequences

Germany) using two-step amplification. In the first step, initial denaturation of 5 min at 94 °C was followed by 5 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min. In the second step, 33 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 30 s, and extension at 72 °C for 1 min were followed by a final extension step at 72 °C for 10 min. Se-

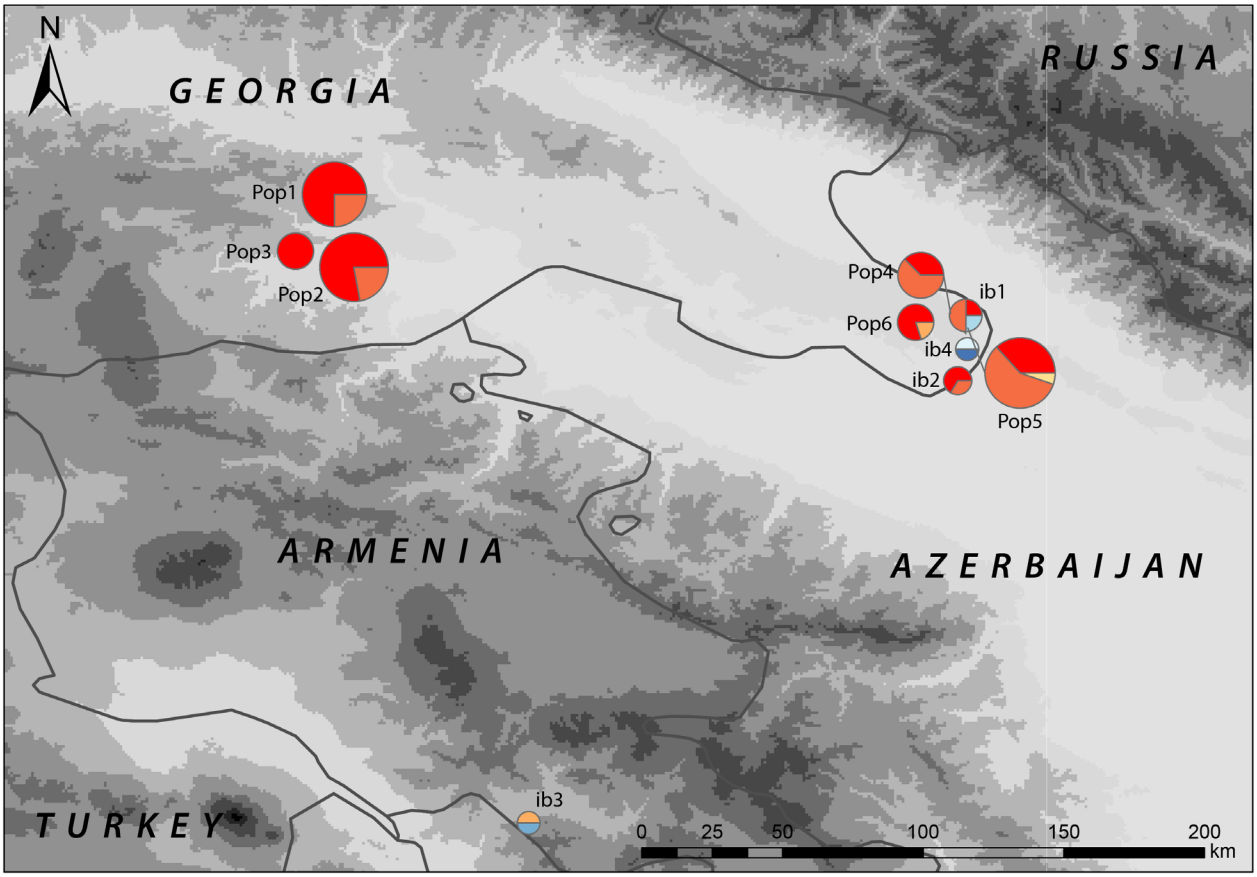

Figure 1. Distribution of the studied populations and ribotypes of *Acer ibericum* in Georgia; colour coding of the ribotypes refers to Figure 2 (population codes as given in Table 1 are next to the ribotype pie-charts)

https://doi.org/10.17221/36/2021-JFS

quencing was accomplished on both strands using a 3730 DNA analyzer (Applied Biosystems, USA) by the laboratory centre of the Senckenberg Biodiversity and Climate Research Centre Frankfurt (SBiK-F) with the primers used for the PCR.

ITS data analyses. Sequences were assembled using Geneious v11.1.4 (Kearse et al. 2012), aligned using MAFFT v7.388 (Katoh, Standley 2013). Newly generated sequences are deposited in NCBI GenBank (Table S1 in Electronic Supplementary Material (ESM), accession numbers pending).

ITS sequences with coded indels were analyzed using the TCS statistical parsimony algorithm (Clement et al. 2002) as implemented in PopART v1.7 (Leigh, Bryant 2015) with masked nucleotide positions with undefined states. Haplotype (ribotype) diversity (*h*) (Nei, Tajima 1983) and nucleotide diversity (π) (Nei 1987) of populations were calculated using DnaSP v5.10.1 (Librado, Rozas 2009). Ambiguous nucleotide positions were harmonized according to the corresponding ribotypes before the calculation in DnaSP.

BLAST analyses. BLAST mediated species identification was performed in Geneious. Database search was done by pairwise comparison using the MegaBLAST algorithm (Zhang et al. 2000) with the use of the following settings: scoring (match mismatch): 1–2, gap cost (open extend): linear, max E-value: 10; word size: 28. The output was ordered by increasing bit-score for each hit (Table S2 in ESM). As the genus *Acer* was intensively studied in the past and there are several ITS sequences for the majority of the species in the GenBank as well as some interspecific ITS variant sharing, we considered the identification based on the majority of the taxonomic assignments among the top 10 hits with the highest bit-score unless there was a sharp decline in the bit-score.

RESULTS

Sequence analyses. Amplified and quality trimmed ITS sequences were 803–807 bp long resulting in the 810 bp long alignment and comprised full ITS1 and ITS2 introns as well as 5.8S rDNA exon and parts of the 18S and 25S rDNA. The analysis revealed two length variants separated from each other by two indels, three single nucleotide polymorphisms, and an individual (Ai5-19) with an additional nucleotide at position 770. Interestingly, the majority of the samples (62) revealed up to 4.4% ambiguous nucleotide positions and most individuals (46) shared the short length variant. After inclusion of the sequences previously published by Grimm et al. (2007), two indels were manually coded (presence and absence) and the alignment was trimmed to 662 bp. The alignment contained 18 variable sites, of which 6 were parsimony informative. The TCS analysis recovered 8 ribotypes (R1–R8; Figure 1, Table 2) separated from each other by one to two mutations. Most individuals of the studied *A. ibericum* shared the short variant ribotype (R1).

BLAST analyses. MegaBLAST search of studied samples against the GenBank database revealed that all studied accessions could be considered *Acer ibericum*. The short length variant consistently placed *A. ibericum* on top 3 places regarding the

Table 2. Diversity indices of *Acer ibericum* populations

Population	*Nb*	$Nb_{riboTCS}$	$Nb_{riboDnaSP}$	*h* ± SD	π (%) ± SD
Pop 1	16	2	3	0.433 ± 0.138	0.00337 ± 0.00099
Pop 2	18	2	3	0.386 ± 0.128	0.00293 ± 0.00092
Pop 3	5	1	1	0.000	0.000
Pop 4	8	2	2	0.536 ± 0.123	0.00405 ± 0.00093
Pop 5	19	3	4	0.591 ± 0.088	0.00246 ± 0.00031
Pop 6	5	2	2	0.400 ± 0.237	0.00060 ± 0.00036
ib1	4	3	3	0.833 ± 0.222	0.00604 ± 0.00276
ib2	3	2	2	0.667 ± 0.314	0.00403 ± 0.00190
ib3	2	2	2	–	0.01360 ± 0.00680
ib4	2	2	2	–	0.01057 ± 0.00529

Nb – number of studied individuals; Nb_{ribo} – number of ribotypes identified by TCS/DnaSP; *h* – ribo(haplo)type diversity; π – nucleotide diversity; SD – standard deviation

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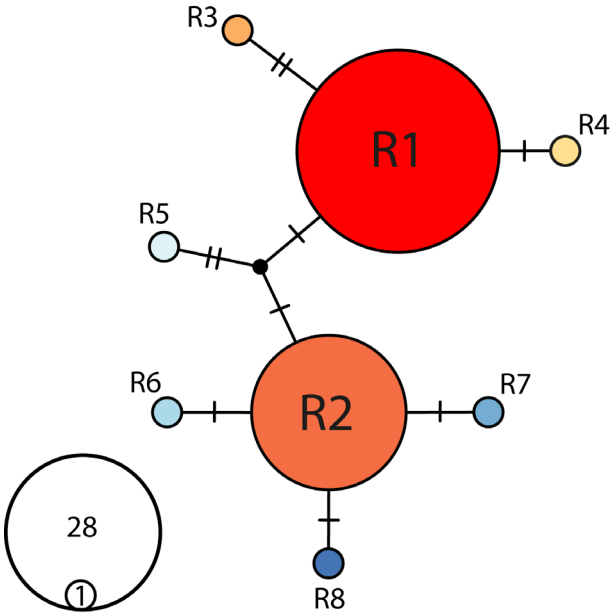

Figure 2. Statistical parsimony network based on ITS sequences of *Acer ibericum*; small empty circles represent ribotypes that are not present, but necessary to link all the ribotypes recorded to the network; all ribotypes are separated from the nearest ribotype by one mutation/indel

bit-score as well as *A. ibericum* presented the majority of the hits (7/10). The only exception was the sample Ai2-2 for which *A. ibericum* was assigned to the top 3 hits, but it did not represent the majority among the top 10 hits. For the long variant (25 samples) the highest bit-score hit was consistently *Acer sempervirens* (GenBank accession AM238350). However, *A. ibericum* was the second (and in six cases the third) best hit and *A. ibericum* also always represented the majority of the top 10 hits (6/10). The details for particular samples are given in Table S2 in the ESM.

Genetic diversity. Haplotype and nucleotide diversities of the populations are summarized in Table 2. The highest values for haplotype and nucleotide diversities were recorded in population 5 (Shavi Mountain) as well as in population ib1 (Alpadara, Vashlovani National Park) when published data are considered.

DISCUSSION

DNA barcoding/identification of *Acer ibericum*. In our study, two ITS variants were obtained for *A. ibericum* that differ in length and to a certain degree in sequence, similarly as found previously (Grimm et al. 2007; Grimm, Denk 2014). Due to the large proportion of ambiguous nucleotide positions our data suggest the presence of both fragments within the majority of the individuals. Accordingly, our data represent only the dominant variant per individual. Unfortunately, cloning was not possible within this study, but a careful check of the positions with ambiguous bases further confirms the presence of both variants. This was previously explained as an ancient polymorphism or potentially an ancient hybrid origin of *A. ibericum* and this idea was supported by the discovery of *A. sempervirens* individuals bearing an *A. ibericum* ITS homeologue as well as by a relatively low number of mutations required for assumed *A. ibericum* sister taxa (Grimm et al. 2007).

Within our sampling, only the short variant was recovered with no evidence of the presence of the second fragment (neither ambiguities nor double peaks in electropherograms) in nine individuals from two populations (Pop 1, Pop 2). This could be explained either (1) by strong disproportion of both variants in the respective individuals due to advanced concerted evolution (e.g. Calonje et al. 2009) or (2) it could represent the ancestral ribotype of *A. ibericum*. In the latter case, the rather recent acquisition of the second fragment could be considered. This is, in our opinion, less probable and would make the presence of *A. ibericum* ITS variant in *A. sempervirens* (distributed mainly in the Aegean) difficult to explain. Nevertheless, as there are reports on artificial as well as natural hybrids among closely related *Acer* taxa (e.g. Johnson 1939), markers with higher resolution and broader sampling including intermediate morphotypes are necessary to further clarify the origin and the evolution of the particular taxa in the series *Monspessulana*.

All individuals with dominant short fragments were clearly assigned to *A. ibericum*, as in all cases the top 3 hits corresponded to this taxon. However, if the proportion of the ambiguous nucleotide positions was relatively high, also other related taxa were recovered among the top 10 hits. Concerning the long ITS variant, *Acer sempervirens* (GenBank accession AM238350) was consistently recovered as the top hit. This is in line with Grimm et al. (2007) as this accession represents the hybrid *A. sempervirens*, bearing the ITS homeologue of *A. ibericum*. Interestingly, although there were additional single nucleotide polymorphisms, the MegaBLAST algorithm identified rather the *A. sempervirens* vari-

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ant than the corresponding and identical *A. ibericum* sequence (GenBank accession AM238354). AM238354 is shorter by more than 50 bp than the other accessions in GenBank, which makes the statistics of the global sequence comparison always less significant. One solution of this problem is to BLAST only the ITS1–5.8S RNA–ITS2 portion of the amplified region, which in our test runs resulted in correct identification. Accordingly, the ITS1–5.8S RNA–ITS2 marker could be used to reliably identify *A. ibericum*.

Genetic diversity and conservation implications. Our study together with the published data recovered 8 ribotypes. However, if we consider new data, only 3 ribotypes were recovered, which means that although our sampling covered the majority of the Georgian distribution of *A. ibericum*, we missed a certain portion of genetic variation in the sampling. On one hand, it could be explained by a focus of this study on Georgia, as two (R3, R7) of the unsampled ribotypes were previously recovered in the material from Armenia (Grimm et al. 2007). This also fits with the observation of dominant ribotypes R1 and R2 in previously studied Georgian populations ib1 and ib2 (Grimm et al. 2007). On the other hand, we did not recover 3 additional ribotypes (R5, R6, R8) found previously in the vicinity of Alpadara, Georgia. Although the population sampling is relatively unbalanced and the diversity recovered by ITS marker is limited, we consider it insightful to preliminarily estimate the diversity parameters for studied populations. Population 5 from Shavi Mountain as well as populations from Alpadara (ib1, ib4) seem the genetically most variable ones, and based on presented data these populations could be prioritized if conservation measures are planned. Interestingly, the Shavi Mountain population (Pop 5) was the largest population suggesting that current local climatic and soil conditions are favourable. On the other hand, the Alpadara population located in Vashlovani National Park (ib1, ib4) is rather in decline and the trees could not be found when collecting in the scope of this project (Goginashvili, pers. obs.). Taking the occurrence of additional ribotypes in Alpadara together with the semi-desert climatic conditions, we may hypothesize that the Alpadara population might represent a part of the primeval gene pool from the Pleistocene when the adaptation of *A. ibericum* to the modern dry and continental climates most probably occurred (Grimm et al. 2007). However, in order to reliably assess the population genetic parameters as well as to identify conservation targets with the aim to capture the most of the genetic diversity of *Acer ibericum*, more detailed population genetic studies using highly variable microsatellite markers are necessary.

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