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PLANT GENETICS

Genetic Structure of the Ni-Accumulating *Alyssum* L. Species (Odontarrhena) in the Urals

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Abstract—A species-level phylogeny of the Odontarrhena section in the Urals was estimated for the first time. *Alyssum obovatum* (C.A. Mey.) Turcz., *A. tortousum* Willd. and *A. litvinovii* Knjaz. are known for their heavy metal hemi- and hyperaccumulating ability. A total of 15 haplotypes were found based on the genetic diversity of the two chloroplast DNA markers. There are only a few species-specific haplotypes observed in the *A. obovatum* and *A. tortuosum* populations, while geographically close populations of both species share the most part of closely related haplotypes. Thus, the species form a geographically structured pattern of haplotype distribution. The Ural endemic *A. litvinovii* turned out to be monomorphic and genetically close to the other species.

Keywords: genetic diversity, cDNA, Urals, hyperaccumulation, *Alyssum*, *trn*S-*trn*G, *trn*H-*psb*A
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INTRODUCTION

Hyperaccumulators are the plants capable of accumulating concentrations of heavy metals hundred or thousand times higher than other plants [1]. *Brassicaceae* family contains about 25% of all known hyperaccumulatimg plant species [2]. Mostly such species belong to *Alyssum* L., *Noccaea* Moench., *Thlaspi* L. genera and they accumulate nickel. Unique ability of these plants is useful for phytoremediation purposes enabling to exclude heavy metals from the environment without the use of chemical or mechanic technics [3]. Most hyperaccumulating species in Brassicaceae family are useful in terms of agricultural and reclamational selection due to high amount of genetic variability they possess [4]. However hyperaccumulating plants aside from biotechnological purposes are valuable model objects for research of microevolutional processes and adaptation to the harsh conditions of the environment. Like so there was the correlation between the level of genetic variability and the ability to accumulate heavy metals in few papers discovered [5, 6], making it relevant to investigate genetic diversity and phylogenetics of this group of species.

The genus *Alyssum* containing more than hundred species is now taxonomically revised. In regard to many European species a huge discrepancy between taxonomic and molecular-genetic, ploidy and morphology based phylogenetic reconstructions has been observed [7–9]. Moreover, a few taxa that previously belonged to *Alyssum* genus in the rank of section Odontarrhena, at the moment are proposed to be separated into a single genus *Odontarrhena* [10]. The taxonomy of this section in the Northern Eurasia is entangled. The spectrum of views on the Odontarrhena's taxonomic diversity could be narrowed into multiple approaches, varying from those with the single polymorphic species *Alyssum obovatum* (=*Odontarrhena obovata*) [11] to two and three species [12], or even eight species only for Asian part of Russia and Mongolia [13]. Thus, the boundaries and the scope of *A. obovatum* are debatable [14].

Three *Alyssum* species from Odontarrhena section jointly grow in the Urals. *A. obovatum* and *A. tortuosum* species are obligate and facultative nickel hyperaccumulators respectively and they have a broad disjunctive Eurasian area [15]. The third species *A. litvinovii* which is considered to be the Urals' endemic [16] has a hemiaccumulative nickel ability [15]. There is a complex of morphologically hard to distinguish plant species that have never been studied before in the territory. Therefore at the initial stage of the research it is essential to arrange the methodology of solving questions about the genetic structure and dynamics of the ranges of *Alyssum* species in the Urals. And also whether there is the genetic diversity of populations or reproductive barriers between those species.

The research is aiming to evaluate the effectiveness of using chloroplast DNA (cpDNA) markers to determine genetic relationships between *A. obovatum*, *A. tortuosum* and *A. litvinovii* species in the Middle and

Table 1. Geographic coordinates of collection sites and identified cpDNA haplotypes in the studied samples of *A. obovatum, A. tortuosum, A. litvinovii, A. lenense*

No.	Population	Coordinates, N/E	*N*	N_h	N_s
1	*A. litvinovii* Or. region, Novokievka	51°28′/58°10′	17	c:17	c1:1
2	*A. obovatum* Bashk., Shigaevo	53°48′/58°11′	17	d:8 c:9	d2:1
3	*A. obovatum* Bashk., Kalkan	54°25′/59°20′	9	d:1 c:8	d1:1
4	*A. obovatum* Chel. region, Egoza	55°45′/60°26′	12	b:12	b1:1
5	*A. obovatum* Chel. region, Vishnevogorsk	55°58′/60°38′	2	c:1 e:1	c4:1
6	*A. obovatum* Chel. region, Gorn. vozdukh	53°42′/58°39′	13	c:13	c2:2
7	*A. obovatum* Chel. region, Karabash	55°46′/60°19′	10	b:10	b2:2
8	*A. tortuosum* Or. region, Novokievka	51°28′/58°10′	24	c:4 f:12 e:7 d:1	f1:2 c3.1:1 f2:1 c3.2:1
9	*A. tortuosum* Or. region, Akkermanovka	51°11′/58°08′	23	a:23	a:1
10	*A. tortuosum* Or. region, Khabarnoye	51°06′/58°06′	18	a:18	a:3
11	*A. lenense* Or. region, Novokievka	51°28′/58°10′	3	g:3	g:2

No.—population number on the map, *N*—sample size, N_h—number of RFLP haplotypes, N_s—number of sequenced samples.

South Urals. Especially the origin of *A. litvinovii* was willing to be elucidated.

A. obovatum, *A. tortuosum* and *A. litvinovii* are perennials which belong to petrophytic-steppe flora. *A. lenense* Adams from Alyssum section that is common for forest communities was chosen as the outgroup. The species from Odontarrhena section we are interested in all have overlapping morphological characteristics and represent semi-shrubs with small leaves, complex inflorescences and single-seeded locules of pods. *A. obovatum* has the widest natural range for the genus: in Eurasia from Eastern Europe to the north of Central Asia, as well as in North-Eastern America [10]. *A. tortuosum* is distributed in South-Eastern Europe, Russia (the Caucasus Mountains, the Urals, Siberia). In the Urals *A. obovatum* is common in the north of Bashkiria, Sverdlovsk and Chelyabinsk regions, whilst *A. tortuosum* grows in the Orenburg region and in the south of Chelyabinsk region. *A. litvinovii* is a rare species known from a single locality from Mount Dyurtel in Orenburg region [16]. The range of *A. lenense* includes Eastern Europe, Russia and Northern China. The occupied ecological niches are also differing from each species. For example, *A. obovatum* occurs on steppe rocks, rocky slopes, and in petrophytic steppes [17], while *A. tortuosum* occurs on limestone and sandstone outcrops, in stony steppes, on gravelly steppe slopes [18] and *A. litvinovii* grows on carbonated serpentinites in a single locality on grassy slopes, *A. lenense* is common for grassy slopes and forests on various types of rocks.

MATERIALS AND METHODS

We analyzed 148 plants from the territory of the Southern and Middle Urals (Orenburg region, Chelyabinsk region, Republic of Bashkortostan), including 6 cenopopulations of *A. obovatum*, 3 cenopopulations of *A. tortuosum*, 1 cenopopulation of *A. litvinovii* and 1 cenopopulation of *A. lenense*. The names and the numbers of cenopopulations are listed in Table 1; their geographical locations are presented in Fig. 1a. Here and after those will be referred as populations in the text for the convenience of reading.

DNA was isolated according to the standard protocol for plant tissues (CTAB method) [19] from fresh leaves. To select the proper molecular markers amplification was carried out with the most commonly used in phylogenetic and population studies universal primers for chloroplast fragments *trn*H-*trn*K, *trn*K1-*trn*K2, *psa*A-*trn*S and *trn*C-*trn*D [20]; *trn*F-*trn*Vr [21], *trn*T-*trn*F [22], *trn*S-*trn*G [23] according to the protocol and temperature profile of PCR recommended by the authors. Genus specific *de novo* primers for *psb*A-*trn*H intergenic spacer were developed based on the complete chloroplast genome of *Alyssum desertorum* (GenBank accession numer KY498535.1) in the online program Primer3Web (version 4.1.0) [24]: *psb*Aa—GAACGACGGGAATTGAACC; *trn*Ha—TAACCGCGCTAACCTTGGTA. Amplification profile was: preliminary denaturation at 94°C for 5 min, in subsequent cycles: denaturation for 45 s, annealing of primers at 60.5°C for 1 min, elongation at 72°C for 2 min, then final elongation for 10 min at 72°C. In total 35 reaction cycles were held.

Then amplification products were restricted with five enzymes: *Hae*III, *Hin*fI, *Kzo*9I, *Taq*I, *Tru*9I. The most variable chloroplast fragments *trn*S-*trn*G and *psb*Aa-*trn*Ha allowed to detect the biggest number of mutations. These fragments combined with the listed restriction endonucleases were used for RFLP-analysis (Restriction Fragment Lengths Polymorphism) of all 148 samples. The fragments *trn*S-*trn*G and *psb*Aa-*trn*Ha of 20 samples including the four species were

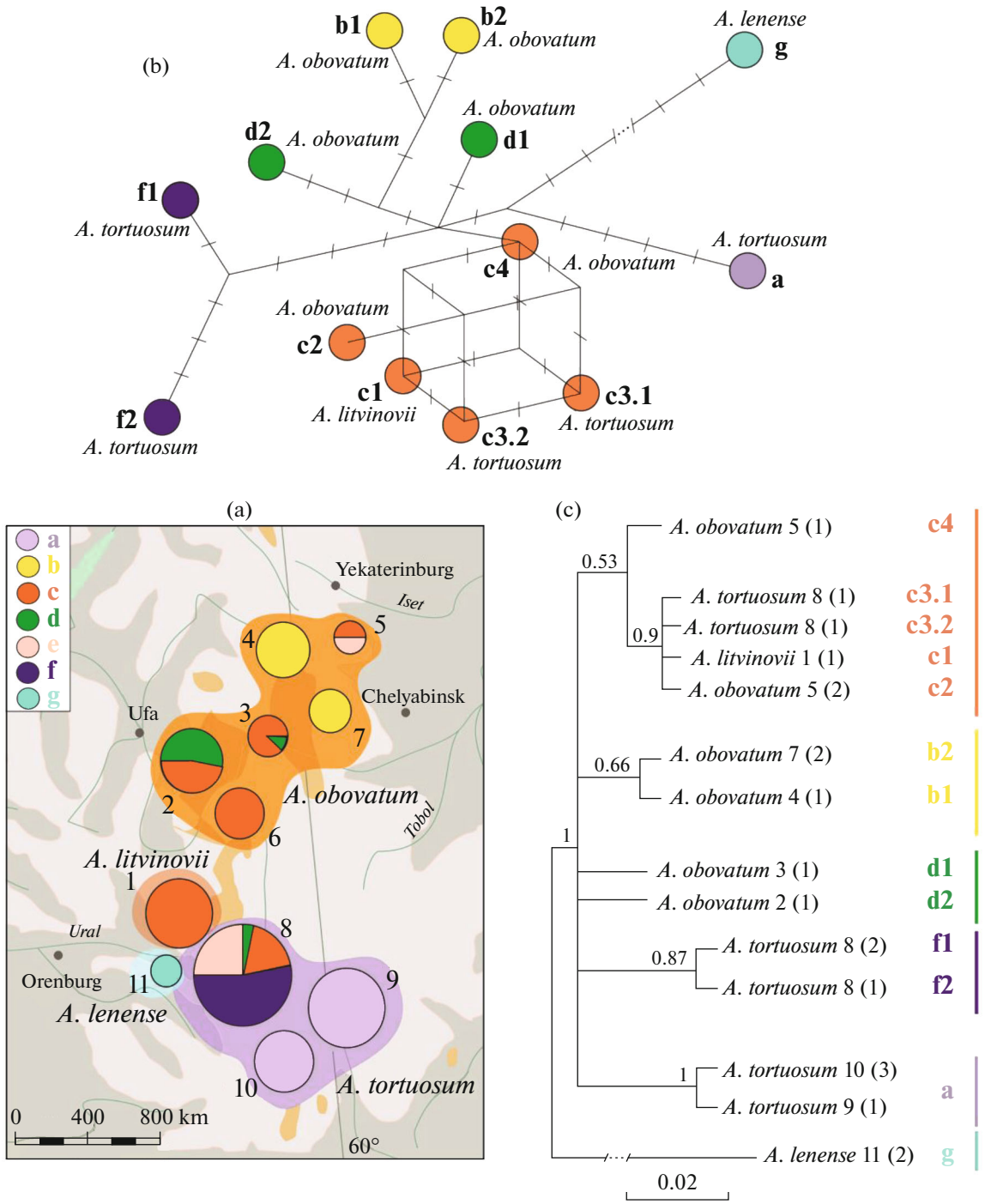

Fig. 1. (a) Location of the studied samples and distribution of detected cpDNA haplotypes, based on RFLP-analysis. The size of the circles is proportional to the sample size. Populations of the same species are united with the shaded area. (b) Median Joining network of cpDNA haplotypes (20 samples). Haplotypes are indicated with colour and index (see explanation in text). Transverse strokes on the branches of a tree are mutational events (not every mutation is indicated for the outgroup specimen). (c) Phylogenetic tree of the studied samples, based on Bayesian analysis. The value of the posterior probability is indicated above the branches; haplotypes are indicated in Latin letters; the numbers correspond to the population numbers in Table 1; the frequency of the haplotype within the sample is indicated in parentheses.

sequenced on an ABI 3130 genetic analyzer (Applied Biosystems, United States). The sequences were aligned manually with the BioEdit software [25]. The phylogenetic tree for all samples was constructed using Bayesian analysis in MrBayes v. 3.1.2 [26] on the basis of the model of nucleotide substitutions GTR + G + I. Insertions, inversions and deletions were considered as single events and were encoded as a binary data matrix

consisting of zeros and ones. Consensus trees were visualized using Fig Tree v1.4.3 program [27]. The haplotype network was built based on the Median Joining (MJ) method in the Network v.5.0.0.3 program [28].

RESULTS

During the first stage of the selection among 24 samples of each of the species the majority of analyzed cpDNA fragments occurred to be monomorphic, moderately variable or unstably amplified. Only two cpDNA fragments turned out to be highly polymorphic. Those are *trn*S-*trn*G and *trn*Ha-*psb*Aa intergenic spacers. At the next stage RFLP-analysis of those two selected cpDNA fragments with all five restriction enzymes was performed on the entire plant material (148 plants). As a result seven chloroplast haplotypes were obtained: **a**, **b**, **c**, **d**, **e**, **f**, **g**. According to the distribution of haplotypes' frequencies (Fig. 1a) it could be concluded that the genetic variability is not species-specific but geographically structured. For example, the southernmost populations *A. tortuosum* 9 and 10 are from Akkemanovka and Habarnoe respectfully and they both share species-specific haplotype **a**. The northernmost populations of *A. obovatum* 4 and 7 are from Egoza and Karabash respectfully and they both share species-specific haplotype **b**. However a common haplotype **c** is distributed through all the three Odontarrhena section species in *A. tortuosum* population from Novokievka (8), in *A. obovatum* populations from Vishnevogorsk (5), Gornii Vozduh (6), Kalkan (3) and Shigaevo (2). Surprisingly, Ural endemic species *A. litvinovii* in population 1 occurred to have only haplotype **c**. Haplotype **d** is common for *A. obovatum* from Shigaevo (2) and Kalkan (3) and for *A. tortuosum* from Novokievka (8). Haplotype **e** is rare and it is observed in *A. obovatum* from Vishnevogorsk (5) and *A. tortuosum* from Novokievka (8). *A. tortuosum* population from Novokievka is the most diverse including samples of four haplotypes (**c**, **d**, **e**, **f**) and the majority of samples have unique **f** haplotype. Outgroup *A. lenense* samples from population 11 have separate haplotype **g** (95 mutations). Thus three of seven haplotypes are shared between analyzed populations.

Sequencing of Ural *Alyssum* samples contributed to a more detailed representation of its genetic diversity. GenBank accession numbers for submitted sequences are OK329970–OK329993. The length of concatenated sequence was 1196 nucleotides (*trn*S-*trn*G 1–688; *trn*Ha-*psb*Aa 689–1196). There were indicated 87 SNPs, 34 indels and 3 inversions in analyzed sequences. Only 8 variable sites were parsimony informative from 124 in total. Intra-specific and intra-population variability was found. Sequencing allowed enlarging the amount of haplotypes from 7 to 15 comparing to RFLP analysis. The pattern of variability distribution remained the same having a strong geographical structure, while RFLP-haplotypes formed clusters of sister haplotypes, that have 1–3 mutations.

Median network relating the cpDNA haplotypes revealed 5 haplotypes pool (**c1**, **c2**, **c3.1**, **c3.2** and **c4**), consisting of common for Orenburg and Chelyabinsk regions haplotype **c**. During RFLP-analysis *A. obovatum* from Gornii Vozduh (6) and *A. litvinovii* from Novokievka (1) used to have the same haplotype **c**. Sequencing allowed to divide them into two single haplotypes: **c1** for *A. litvinovii* and **c2** for *A. obovatum*. *A. tortuosum* from Novokievka (8) showed intra-population variability and split into **c3.1** and **c3.2**. *A. obovatum* from Vishnevogorsk (5) has **c4** haplotype.

A. obovatum specific haplotype **b** divided into haplotype **b1** from Egoza (4) and haplotype **b2** from Karabash (7), while *A. tortuosum* specific haplotype **a** did not divide. *A. obovatum* haplotype **d** turned out to differ in Kalkan (3)—**d1** and in Shigaevo (2)—**d2**. Haplotype **d** as well as haplotype **e** was not sequenced. *A. tortuosum* unique haplotype **f** divided into **f1** and **f2**. Thus sequencing allowed enlarging the picture of *Alyssum* Ural populations' variability.

Bayesian analysis model is shown in Fig. 1c. The tree backbone has four clades, which is in agreement with the number of common haplotypes detected due to RLFP-analysis. The tree's clades obtained from *A. tortuosum* species specific haplotypes **a** and **f1**, **f2** have high statistical support from posterior probabilities (PPvalue 0.87–1.00).

The tree's clade containing haplotype **c** pool united all three species. Its subclades correspond to different populations. Other clades have only limited statistical support. However each of them contains a single species and has a distinctly regional structure within the Urals.

DISCUSSION

Observed picture coincides with the situation when the supposed species are not completely reproductively isolated yet, they hybridize and thus tied with a significant genetic flow. Researchers tend to [29] consider the present area of Odontarrhena section species as the consequence of the rapid expansion of the species after the glaciations. That is why we may suppose that this taxonomic group is exposed to the process of lineage sorting [30–32]. That is why although Ural species already have species specific haplotypes such as **a**, **b**, **f**, however, they may still frequently share the same or sister haplotypes due to incomplete lineage sorting. Further rare variants could be eliminated, whilst sister haplotypes could accumulate a significant amount of mutations, which will lead to the fixation of species specific chloroplast lineages. For example, distinct variants in group **c** haplotypes have already got isolated, and haplotypes **c1**, **c2** and **c3** belongs to *A. litvinovii*, *A. obovatum*, *A. tortuosum* respectively. Ural endemic species *A. litvinovii* should be precisely

observed. It has one of sister haplotypes from group **c** uniting all three species which probably corresponds about its recent origin and close relations with two other species. However at this point it is impossible to tell whether this species possesses limited genetic variability or the samples amount was too small (17 in total). Interestingly *A. litvinovii* has an increased ploidy (according to A.Y. Teptina's unpublished data). Thus its hybrid origin is easy to suppose and it is a very common issue for *Alyssum* genus [33]. Generally, the distribution of revealed variability is in accordance with the data of European species of *Alyssum*. It has been shown [29, 34–36] that molecular markers of different origin from chloroplast DNA to nuclear DNA both ITS and multilocus analysis do not result in phylogeny coinciding with traditional taxonomic species. It is common for endemic species or species having a small range to belong inside a clade of species with broad range at the level of intra-population variability.

The species of Brassicaceae family is frequent in floras originating from heavy metal rich soils. Plants growing in heavy metal containing soils can possess increased genetic diversity [6, 37, 38]. Genetic diversity of plants and the content of heavy metals in soil or the ability to accumulate them in plants are directly connected only in a few papers for *A. bertolonii* from Italy and two species *A. murale* and *A. daghestanicum* from the North Caucasus [5, 6]. Variability of the level of Ni-content in soil is correlated with the value of genetic diversity of population of *A. bertolonii* according to cpDNA microsatellite analysis [38]. *A. murale* populations turned out to be polymorphic in ITS markers and the difference coincided with Ni-accumulating ability of populations. It has been shown that *A. murale* hyperaccumulating population and non-hyperaccumulating population differed genetically in 5 mutations and 16 polymorphic loci, while *A. daghestanicum* with no ability to accumulate Ni was genetically monomorphic [6]. These patterns are true for Ural species variability, because capable of obligate or facultative Ni-accumulation *A. obovatum* and *A. tortuosum* occurred to be more variable than Ni-hemiaccumulator *A. litvinovii*. However data based on variability of cpDNA markers could only indirectly reflect adaptive capabilities of species. To elucidate those patterns nuclear multilocus markers would be more appropriate. For example, species from Odontarrhena section in Albania differed in the amount of outlier loci depending on whether they grew on serpentine soils or not [36]. The more outlier loci (those that do not fit in variability distribution under neutrality conditions) they had, the more potentially adaptive they are.

cpDNA markers that are promising for revealing spatial-genetic structure of *Alyssum* species were detected. All three species are closely related. *A. obovatum* and *A. tortuosum* are genetically diverse and have species specific haplotypes and *A. litvinovii* is lack of genetic diversity. To form more detailed picture of these species' phylogeny, enlarged plant samples, broader area of collecting and more diverse molecular markers will be needed. cpDNA markers could only be effective in case of sequencing which reflects the whole range of variability. Otherwise, RFLP-analysis is more suitable for population studies and it enables to preliminary estimate genetic variability and population structure, although RFLP-analysis has the advantage of lower cost.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest.

This article does not contain any research using animals or people as objects of research.

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