PLANT GENETICS

High Polymorphism and Autotetraploid Origin of the Rare Endemic Species *Oxytropsis chankaensis* Jurtz. (Fabaceae) Inferred from Allozyme Data

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**Abstract**—Using starch electrophoresis, we examined 19 enzyme systems presumably controlled by 35 loci in the rare endemic tetraploid species *Oxytropsis chankaensis* Jurtz. (Fabaceae). Electrophoretic patterns and their genetic interpretation are presented. The isozyme data suggest tetrasomic inheritance in *O. chankaensis*. Three or four alleles at a particular locus were found in a number of individual plants, which indicate the autotetraploid origin of this species. Seventeen loci were shown to be polymorphic. As reliable gene markers for population systems, we recommend highly active polymorphic systems showing good allozyme separation (*Ce-2, Gpi-2, Gpt-2, Idh-2, Lap, Mdh-2, and Mdh-3*). Parameters of allozyme variability proved to be very high for a rare species with a restricted range: \( P = 48.6\%, A = 2, A_p = 3.06, H_{ob} = 0.173 \).}

**INTRODUCTION**

*Oxytropsis chankaensis* Jurtz. is a herbaceous perennial plant from the family Fabaceae, a rare endemic species recorded in the regional list of rare plants [1] and Red Book of Primorski krai [2]. This species is considered tetraploid with \( 2n = 32 \) [3, 4], has a narrow ecological niche (open sands), and grows in small isolated populations on sand dunes and shoals of the western coast of Khanka Lake (Primorski krai). In recent years, *O. chankaensis* has been subject to increasing anthropogenic pressure (road construction, cattle pastures, recreation) nearly over its whole range, which resulted in a dramatic reduction of the population size (A.B. Kholina, personal observations). The development of a strategy of conservation and restoration of the species genetic diversity requires evaluating intra- and interpopulation variability, differentiation of isolated population, and gene flow between them. Our preliminary studies of allozyme variation in *O. chankaensis* [5] revealed an unexpectedly high level of polymorphism in this species with narrow distribution existing in limited environments and experiencing anthropogenic impact on the most part of its range. The cytologically established tetraploid origin of *O. chankaensis* may have a key significance for explaining the observed level of genetic polymorphism. However, to clarify the effect of the doubled genome on the variability and its distribution within and among *O. chankaensis* populations, the polyploidy type of the species should be established.

There are two main types of polyploidy: autopolyploidy (an intragenomic mechanism underlying an increase in the number of monoploid genomes, the presence of several sets of the same chromosomes and genes) and allopolyploidy (intergenomic mechanism providing combination of the genomes of different species and genera via hybridization). Genetic analysis shows substantial differences between auto- and allopolyploids [6, 7]. Allopolyploids exhibit disomic inheritance and fixed heterozygosity due to combination of different genomes. Aurotetrploidploids are characterized by tetrasomic inheritance; in the case of higher ploidy, polysomic inheritance is observed. Autotetrploids often show higher range of variation and higher heterozygosity than diploids; in addition, segregation rate of rare alleles in the former is significantly lower than in the latter [6–8].

The aim of the present study was determining the polyploidy type and allozyme variability parameters in *O. chankaensis* by means of electrophoretic analysis of isozymes as molecular-genetic markers. The data obtained may contribute to understanding the mechanisms of genetic variation maintenance in rare species and provide a basis for developing a strategy of conserving and restoring the species gene pool.

**MATERIALS AND METHODS**

Natural populations of *O. chankaensis* were examined. In all, we studied 154 adult generative plants. The plants were collected in different localities over the total range of the species. As the material for isozyme analysis, we used leaves (fresh or stored in liquid nitrogen). To preserve the enzymes in native state, extraction and all subsequent procedures were carried out at 0–5°C. The enzymes were extracted immediately before electrophoresis by homogenization of leaf tissue (~100 mg) in liquid nitrogen with addition of 200 μl of...
Polymorphic Loci

AAT. The fastest zone presumable controlled by locus Aat-1 had four electrophoretically differing variants (Fig. 1). Heterozygotes had three-band phenotype characteristic of enzymes with dimeric structure (Fig. 2a, lanes 5–8; Fig. 3a, lanes 3, 4, 14). In some plants, this locus was represented by heterozygotes with varying gene dosage effect (Fig. 2a, lanes 10–15; Fig. 3a, lanes 2, 5, 8, 9, 13). The staining intensity distribution in this case corresponds to that expected with tetrasomic codominant inheritance of the alleles. Some plants exhibited a five-band pattern, which indicated the presence of three alleles at the locus (Fig. 2a, lane 9; Fig. 3a, lanes 1, 6). Five rather than expected six bands (products of three alleles and their three intralocus heterodimers) were observed in gel, because the product of heterodimer Aat-12/4 coincides in mobility with allozyme Aat-13.

ACO. Observed on zymograms as one variable band encoded by a low-polymorphic locus with two alleles (Fig. 1). The pattern character with marked gene dose effect corresponds to that observed with tetrasomic inheritance of two alleles for a monomer enzyme. The allele coding for the faster fraction occurs only in heterozygotes.

CE. Polymorphic fraction groups encoded by at least two loci, Ce-1 and Ce-2, were clearly revealed (Fig. 1). The Ce-2-encoded enzyme had the highest activity. Double fractions of heterozygotes for Ce-1 and Ce-2 suggest a monomorphic structure of both esterases. The presence of three- and four-band heterozygous phenotypes is characteristic for autotetraploids having the corresponding allele number. In some cases, the intensities of bands were different, which indicates the presence of unbalanced heterozygotes.

DIA. The slow fraction was represented by an isozyme controlled by a diallelic polymorphic locus Dia-2. The presence of double-banded patterns of heterozygotes indicates that the enzyme is monomorphic. Homozygotes were observed only occasionally. The segregation of allotypes suggested tetrasomic inheritance of alleles at this locus.

FDH. We found one activity zone with rather low staining intensity controlled by locus Fdh with two alleles (Fig. 1). In heterozygotes, three activity fractions (characteristic of dimeric structure) and variable gene dosage effect were observed (Figs. 2b, 3e), which indicates tetrasomic inheritance.

FE. We determined three FE loci: two polymorphic loci Fe-1 and Fe-2, and a monomorphic locus Fe-3. Double fractions of heterozygotes suggest that both polymorphic esterases have monomorphic structure. The fast zone (Fe-1) consisted of different electrophoretic variants encoded by six codominant alleles (Fig. 1), which included three- and four-band phenotypes, patterns with different staining intensities characteristic of autotetraploids. The same picture was observed in the
zone controlled by locus Fe-2 with four codominant alleles.

GPI. Slow polymorphic zone presumably controlled by the Gpi-2 locus (Figs. 2c, 3b). The presence of triple fractions in the overwhelming majority of heterozygotes confirms the dimeric structure of this enzyme. Only in three out of 154 plants examined, the rare Gpi-2a allele was found; note that it occurred in heterozygotes with three alleles and corresponding five-band phenotype (Fig. 2c, lane 10; Fig. 3b, lane 3). The simultaneous presence of three alleles at one locus in one individual plant and the presence of unbalanced heterozygotes of different types on zymograms are characteristic of autotetraploids.

GPT. The enzyme was revealed on zymograms as two activity zones. Polymeric variants with marked gene dosage effect were found in the low-mobility zone (locus Gpt-2), which suggests dimeric structure of the enzyme and tetrasomic inheritance at the locus.

IDH. The slower zones exhibited electrophoretic variants. The slow allozyme of the Idh-2 locus is present in plants at higher frequency; the fast allozyme occurred only in heterozygotes. Heterozygotes exhibited variation in staining intensity of allelic fractions.
Fig. 2. Schematic representation of some electrophoretic variants detected in natural populations of the tetraploid species O. chankaensis and their genetic interpretation. (a) locus Aat-1: lanes 1–4, homozygotes; 5–8, balanced heterozygotes; 9, three-allele variant; 10–15, unbalanced heterozygotes. (b) locus Fdh: lanes 1, 2, homozygotes; 3, 4, unbalanced heterozygotes; 5, balanced heterozygote. (c) locus Gpi-2: lanes 1–3, homozygotes; 4, 7, 8, balanced heterozygotes; 5, 6, unbalanced heterozygotes; 9, 10, three-allele variants. (d) locus Idh-2: lane 1, homozygote; 2, 4, unbalanced heterozygotes; 3, balanced heterozygote. (e) locus Mdh-1: lane 1, balanced heterozygote; 2, 3, unbalanced heterozygotes; 4, three-allele variant; (f) locus Mdh-2: lane 1, homozygote; 2, balanced heterozygote; 3, 4, unbalanced heterozygotes. (g) Locus Mdh-3: lanes 1, 2, homozygotes; 3, balanced heterozygote; 4, 5, unbalanced heterozygotes.

corresponding to the tetrasomic diallelic codominant genetic determination (Figs. 2d, 3d).

**LAP.** We found one polymorphic, high-activity zone controlled by locus Lap with three alleles. The pattern of variation confirms monomeric structure of this enzyme. The presence of types of heterozygotes characteristic of tetraploids (unbalanced, balanced, with simultaneous presence of three alleles) indicates tetrasomic inheritance of the enzyme.

**MDH.** Four activity bands were clearly seen on the zymograms: three high-activity zones encoded by polymorphic diallelic loci Mdh-1, Mdh-2, and Mdh-3 (Figs. 2e–2g; Fig. 3e) and slow low-activity zone controlled by monomorphic locus Mdh-4. Interpreting zymograms in zones controlled by polymorphic loci was rather problematic. The fastest zone was mainly represented by three-band heterozygous phenotypes showing gene-dosage variation (Fig. 2e, lane 4; Fig. 3e, lane 14) corresponding to heterozygotes with three alleles, in which the product of allele Mdh-1 represents mobility with the fast allozyme of the Mdh-2 locus, and the third fraction contains the products of the Mdh-1 allele and the intralocus heterodimer Mdh-1/2. In addition, the slow Mdh-2 allozyme coincides in mobility with the fast allozyme of the Mdh-3 locus. The pattern was clarified by the presence of phenotypes of homozygotes Mdh-2/2 and Mdh-3/3 (Figs. 2f, 2g; Fig. 3e, lanes 3,
8, 9, 12). Gene dosage effect characteristic of tetraploids was also observed for loci Mdh-2 and Mdh-3. The polymorphic variants suggested dimeric structure of the enzymes.

PGM. Two activity zones were found, which are presumable controlled by two polymorphic loci, Pgm-1 and Pgm-2 (Fig. 1). Locus Pgm-1 exhibited several electrophoretic variants controlled by four codominant alleles;
homzygotes of all types, balanced and unbalanced heterozygotes with alleles in different combinations, and heterozygotes with simultaneous presence of three alleles of different staining intensity depending on gene dosage. In the zone controlled by the Pgm-2 locus Pgm-2\textsuperscript{1} homzygotes occurred at highest frequency, while heterozygotes, duplexes and triplexes for this allele were less frequently observed. The slow allozyme occurred only in heterozygotes. Judging by the double fractions of heterozygotes, this enzyme is a monomer.

In all, we examined 19 enzyme systems in O. chankaensis. Eight of them—AAP, ACO, ADH, ALD, FDH, G-3PDH, HK, and LAP—presumably have one locus whereas the remaining systems are multilocus. Loci Aat-1, Aco, Ce-1, Ce-2, Dia-2, Fdh, Fe-1, Fe-2, Gpi-2, Gpt-2, Idh-2, Lap, Mdh-1, Mdh-2, Mdh-3, Pgm-1, and Pgm-2 were shown to have allelic variants. In total, we found 70 alleles at 35 isozyme loci in the samples examined.

Out of the enzyme systems studied, we selected high-activity polymorphic systems presumably encoded by seven loci (Ce-2, Gpi-2, Gpt-2, Idh-2, Lap, Mdh-2, Mdh-3). They are easily and reliably detected in starch gel showing good separation and thus can be recommended as gene markers for studying the population-genetic structure of O. chankaensis.

Allozyme variation parameters in the examined species proved to be quite high: $P = 48.6\%$, $A = 2$, $A_p = 3.06$, $H_{ob} = 0.173$.

**DISCUSSION**

The results of isozyme analysis confirm the tetraploid nature of O. chankaensis [3, 4]. The presence of balanced and unbalanced heterozygotes for all polymorphic loci and heterozygous variants showing three or four alleles at each locus indicate tetrasomic inheritance. Collectively, such data generally suggest that the species is an autoploidy [7, 8, 14].

The estimation of polymorphism in such species has some specific features. Autotetraploids are expected to have more polymorphic loci, alleles per locus and higher heterozygosity than its possible progenitors, closely related to it diploid species (or tetraploid and diploid races within the species) [15, 16]. This is shown in most studies concerned with comparative analysis of allozyme polymorphism of polyploid species and their diploid progenitors [7, 14, 17, 18]. For instance, Mahy et al. [14] found in tetraploid populations of Vaccinium oxyccocos, 15 alleles not detected in the diploid cytoype, and the allozyme variation parameters were shown to be significantly higher in the tetraploid than in the diploids [14]. The authors think that the presence of numerous unique alleles is caused by reduced effects of genetic drift in autotetraploids populations.

Although autotetraploids and closely related diploids in some cases are very similar genetically, do not differ in the number of polymorphic loci, have nearly identical allele sets at all loci, and share most common alleles, heterozygosity of tetraploids is always higher than that of diploids [8, 19–21]. High heterozygosity values in such cases are directly associated with tetrasomic genetic structure rather than with the presence of alleles absent in diploids.

As to allozyme polymorphism of narrow-range or endemic tetraploids and their putative progenitors, its level depends on a number of factors. If the putative ancestor is a diploid with restricted distribution, the derived tetraploid endemic exhibits fairly high polymorphism, as was shown for Antennaria species [17, 18]. Variation parameters, including heterozygosity, of a widely distributed diploid ancestor may be higher than in the tetraploid with a narrower range, as it was found in a comparative study of variation in autotetraploid Deschampsia mackenzieana and closely related diploid species D. cespitoosa [22]. This is explained by the founder effect, restricted gene flow between the parental and newly appeared populations as well as by the fact that the endemic tetraploid species arose from a single polyploidization event.

If the ancestor of the narrow-range tetraploid also has a double chromosome set, two scenarios are possible. For instance, Purdy et al. found that two tetraploids—widespread Stellaria longipes and endemic S. arenicola—both exhibited a fairly high level of polymorphism, but all indices were lower in the endemic species [23], which commonly occurs in comparisons of narrow- and wide-range species of the same ploidy level [24]. Comparing two autotetraploids Iris species, Hannan and Orick [25] detected strikingly high variability in the widespread species I. cristata ($P = 73.3\%$, $A = 3$, $A_p = 3.73$, $H_{ob} = 0.231$) and the total absence of allozyme polymorphism in endemic I. lacustris [25]. As a possible explanation, the authors invoked the recent origin of I. lacustris from a genetically depleted population that had passed through a bottleneck. Finally, Purdy and Bayer [26] showed that all variation indices of the sand-dune endemic Achillea millefolium ssp. megacephala were higher than in the widespread autotetraploids species A. millefolium ssp. lanulosa, which is explained by the authors by a transition of the endemic species to obligate sexual reproduction [26].

The allozyme polymorphism in O. chankaensis ($P = 48.6\%$, $A = 2$, $A_p = 3.06$, $H_{ob} = 0.173$) is very high as compared to that of other autotetraploids species examined. This fact has several explanations related to the mechanism of polyploid formation and consequences of doubling the genome of this species.

Stebbins [15] suggested that natural autoploids probably arise through hybridization between individual plants rather than doubling of the chromosome set of a single plant. Autoploids originated from one plant cannot have more than two alleles at each locus, whereas autoploids that appeared via hybridization of two different plants may bear up to four alleles at a single locus. In O. chankaensis, nine out of seventeen
polymorphic loci (Aat-1, Ce-1, Ce-2, Fe-1, Fe-2, Gpi-2, Lap, Mdh-1, Pgm-1) had three or four alleles. This suggests the following: (1) autotetraploids O. chankaensis arose through a cross between two genetically different plants by combination of unreduced gametes; (2) the chromosome sets in individual O. chankaensis plants were repeatedly doubled with subsequent crossing of individual autotetraploids. Regardless of which of the hypotheses is more plausible, “hybridization” according to Stebbins apparently took place, whereas the high level of polymorphism found in O. chankaensis indicates that polyploids resulted from recurrent crosses.

Autopolyploidy leads to elevated heterozygosity and considerable diversity of enzyme forms referred to as “multiplicity” of enzymes [6, 15]. For instance, if in populations of diploids, individuals with maximum three isozymes at a locus (for dimeric enzymes) occur, up to ten isozymes at the locus can be observed in one individual of a tetraploid population. Enzyme multiplicity results in enhanced biochemical variability of a plant; the newly arisen enzyme forms may have novel properties, higher enzyme activity or a special function leading to new types of regulation [6, 16]. Diversity of isofoms can in general improve coordination of metabolic processes, which, in turn, allows polyploids to maintain a high level of metabolism even in a changing environment [15, 16]. Evolutionary success and ecological adaptation of autopolyploids may be directly related to high heterozygosity and enzyme multiplicity.

Multiplicity of enzymes in O. chankaensis is apparent. However, based on high indices of polymorphism at the species level, one cannot adequately evaluate the condition of individual populations and the diversity of their gene pools. Our earlier preliminary study of allozyme variation [5] suggested the presence of unique allele sets in some populations, which provides very helpful information for reintroduction and conservation measures. Data on the age structure, population size and productivity, and the level and distribution of intra- and interpopulation variability are also of much importance. Further research of biology and genetics of O. chankaensis populations are required in order to evaluate their condition. Moreover, to estimate the basic level of polymorphism of O. chankaensis and the contribution of its polyploid origin, comparative analysis of this species and its putative diploid ancestor is required.

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REFERENCES


